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VOLUME 7

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Annual Review of
CHRONO PHARMA COLOGY

Editors: A REINBERG
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ANNUAL REVIEW OF CHRONOPHARMACOLOGY

Volume 7

BIOLOGICAL RHYTHMS AND MEDICATIONS

Proceedings of the Fourth Conference of Chronopharmacology, Nice, 12–15 March 1990

Editors:

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Pergamon Press, Inc.
Maxwell House Elmsford, NY 10523
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FOREWORD

The Annual Review of Chronopharmacology is a publication of the Association des Rythmes Biologiques et Médicaments (Association of Biological Rhythms and Medications). The goal of this series is to provide a scholarly means of rapidly communicating new developments in the field of chronopharmacology. Even-numbered volumes consist of in-depth review chapters authored by investigators well known for their expertise in the field. The selection of the topics and contributors to these volumes is guided by the Scientific Committee of the Association. Odd-numbered volumes contain the Proceedings of the International Conferences of Chronopharmacology which are organized by the Association. This volume, number 7 of the series, contains papers which were presented at the Fourth International Conference on Biological Rhythms and Medications held in Nice, France, 12 -15 March 1990 and which passed peer review by members of the Scientific Committee. The objectives of the International Conferences of Chronopharmacology are: 1) to provide a forum for the presentation and discussion of new findings, methods and concepts in chronopharmacology and chronotherapeutics, 2) to promote sound research involving biological rhythms and medications, 3) to build bridges between pharmacologists, chronobiologists and physicians working in the pharmaceutical industry, in academia and in clinical medicine, and 4) to stimulate the application of findings in medicine.

The Fourth International Conference of Chronopharmacology, which was sarctioned the Le Centre National de la Recherche Scientifique, the Fondation Adolphe de Rothschild, the International Union of Pharmacology, the International Society of Chronobiology, the European Society for Chronobiology, and the Société de Chronobiologie de Langue Francaise, hosted 150 participants from 15 different countries. The published Proceedings compiled in this volume deal with seven major themes. These are outlined below along with the chairpersons of each session.

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SECTION 1

NEUROBIOLOGY

Chairpersons. J. Arendt and C. Englund

CIRCADIAN RHYTHM IN SEROTONIN RELEASE FROM HIPPOCAMPAL NEURONES OF THE RAT: ROLE OF TERMINAL AUTORECEPTORS

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ABSTRACT

The marked circadian variation in serotonin (5-HT) concentrations which occurs in many areas of the brain appears to result from circadian rhythms in both synthesis and release of the transmitter. In this paper we have investigated the hypothesis that in the hippocampus of the rat the circadian rhythm in 5-HT release is generated or controlled by the terminal $5-HT_{1B}$ autoreceptor. No circadian variation in the response of the autoreceptor to either the agonist 5-HT or the antagonist methiothepin was found. These results suggest that changes in sensitivity or coupling of the autoreceptor do not play a significant role in controlling the circadian rhythm of transmitter release.

KEYWORDS

5-HT, circadian, autoreceptors, hippocampus, methiothepin.

INTRODUCTION

A circadian rhythm in the concentration of 5-hydroxytyptamine (5-HT) in rodent brain is well established (Quay, 1968; Hillier and Redfern, 1977). Although the phase of the rhythm of 5-HT concentrations is not the same in all brain regions, the peak concentrations occur during the light phase (Quay, 1968). The observed rhythm appears to be the resultant of several factors; thus while the rate of synthesis of 5-HT is highest during the light phase (Hery et al., 1972), release is greatest during the dark phase (Martin and Marsden, 1985), when the animals are most active. Release of 5-HT is, in turn, affected by many factors, including the activity of the terminal autoreceptors which, when stimulated by 5-HT, inhibit transmitter release (Moret ,1985).

In the rat these terminal autoreceptors are of the $5-HT_{1B}$ subtype (Engel et al., 1986). In previous studies of the behavioural sequelae of 5-HT receptor stimulation we have demonstrated that responses dependent on $5-HT_2$ receptor stimulation display a

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consistent circadian variation while those dependent on 5-HT$_1$
receptors do not (Moser and Redfern, 1984; 1985; 1986).

In this paper we have examined the hypothesis that changes in
activity at terminal autoreceptors are responsible for generating
or controlling the circadian rhythm in the release of 5-HT from
serotoninergic neurones.

METHOD

Male Wistar rats (University of Bath strain) were housed in groups
of 6 under controlled conditions of light, sound and temperature
(Hillier et al., 1973). All animals were acclimatised to a 12:12
light-dark cycle for at least 14 days prior to use, at which time
they weighed approximately 300g. The hippocampus was rapidly
dissected from two freshly killed rats and chopped in two
directions at 250μM on a McIlwain brain tissue chopper. The slices
were incubated for 15 mins at 37°C in Krebs buffer containing 0.1μM
tritiated 5-HT and 10μM pargyline. After 3 washes with 5ml Krebs
buffer, 25μl aliquots of slices were transferred to each chamber of
a superfusion apparatus.

After a 30 min superfusion with
Krebs containing the 5-HT
uptake inhibitor paroxetine
(3.2μM), the medium was changed
to isosmotic modified Krebs
solution containing 25mM
potassium ions (a concentration
shown to evoke a sub-maximal
release rate, Fig.1), and 3.2μM
paroxetine. The potassium
evoked release of tritium was
measured by liquid
scintillation counting in
samples collected every 4 min.
Cumulative dose-response curves
to unlabelled 5-HT were
constructed using 4
concentrations of the agonist
(30nM to 1000nM). Antagonist
experiments were performed by
the addition of 1μM
methiothepin to the superfusion
medium, concurrently with the
elevated potassium. The
antagonist was present
throughout the rest of the
superfusion. Statistical
analysis was carried out using
Student's t-test.

Fig.1. Effect of varying concentrations of
potassium on the release of [^{3}H] 5-HT from
slices of rat hippocampus. Results, (Means ±
s.e.m.) are expressed as fractional release
rate.

RESULTS
The substitution of normal
Krebs solution by Krebs
containing 25mM potassium ions
caused an increased efflux of
tritium from slices of rat
hippocampus. This increase was
shown to be entirely Ca^{2+}
dependent (Fig. 2). The
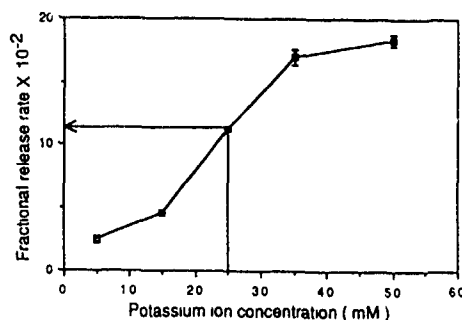
Fig.2. Calcium dependence of the release of
[^{3}H] 5-HT from slices of rat hippocampus,
evoked by potassium. Results, (Means ±
s.e.m.) are expressed as fractional release
rate.
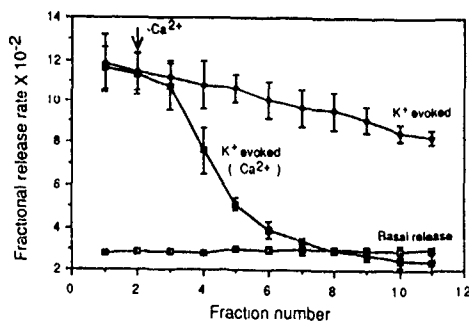
cumulative addition of unlabelled 5-HT caused a dose related
inhibition of tritium release (Fig. 3). Apparent pIC_{50} values
($-log_{10}$ of the concentration of 5-HT required to inhibit the
potassium evoked release by 50%) were calculated from experiments
conducted at four equally-spaced time points in the light-dark
cycle (Table 1). Methiothepin caused a dose related attenuation of
the effect of 5-HT (Fig. 4).

Fig.3. Dose response relationship of the
effect of 5-HT on the [^3H] 5-HT efflux from
rat hippocampal slices, evoked by continuous
potassium (25mM) stimulation. Results,
expressed as fractional release rate, are the
means ± s.e.m. of 5 experiments.
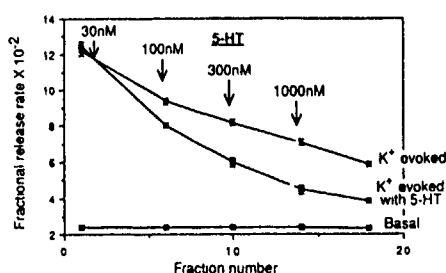
Fig.4. The inhibitory effect of 5-HT on [^3H]
5-HT efflux from hippocampal slices and its
antagonism by methiothepin. The results are
the means ± s.e.m. of 5 experiments.
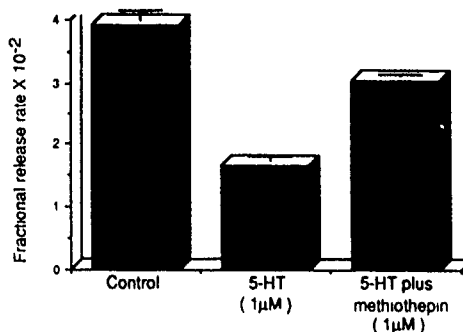
The apparent pA_2 values, calculated at the same time points from
the shift in the dose response curve to 5-HT, are also shown in
Table 1.

Table 1.

Hours after lights on	5-HT apparent pIC_{50} ± sem (n)	Methiothepin apparent pA_2 ± sem (n)
0	7.125±0.063 (5)	6.999±0.045 (5)
6	7.161±0.023 (5)	6.905±0.055 (5)
12	7.024±0.039 (5)	6.846±0.066 (5)
18	7.173±0.047 (5)	6.872±0.108 (5)

Basal tritium release was unaffected by either 5-HT ($1\mu M$) or
methiothepin ($1\mu M$). However, methiothepin did significantly
enhance the potassium evoked release of tritium. This enhancement,
which is generally interpreted as indicating the existence of an
endogenous inhibitory tone, was not significantly different at the
four time points.

DISCUSSION

These results from the hippocampus are in agreement with previous
findings obtained using rat cerebral cortex slices (Singh et al,
1989). They add further support to findings from behavioural
experiments (Moser and Redfern, 1984; 1985; 1986) that receptors of
the $5-HT_1$ subtype do not display a circadian rhythm in response to
stimulation. It has been suggested (Martin and Marsden, 1985) that
the circadian rhythm in the release of 5-HT from central
serotoninergic neurones derives at least in part from a

corresponding rhythm in sensitivity of the terminal 5-HT$_{1B}$ autoreceptors. These results do not support this hypothesis, since they demonstrate clearly that the response of the 5-HT$_{1B}$ autoreceptor to both agonist and antagonist is unchanged throughout the light-dark cycle. Thus the serotoninergic terminal will be susceptible, through the 5-HT$_{1B}$ autoreceptor, to circadian changes in concentration of 5-HT in the synaptic cleft. This and other factors, rather than any circadian rhythm in terminal autoreceptor function, must be assumed to control the observed circadian rhythm in transmitter release.

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LIGHT RESPONSIVENESS OF A CIRCADIAN OSCILLATOR DURING LITHIUM AND POTASSIUM TREATMENT

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ABSTRACT

We generated phase response curves to light (PRCs) in Syrian hamsters fed 1) a
standard hamster diet, 2) diet containing supplemental potassium, or 3) diet with
0.4% lithium carbonate and supplemental potassium. Lithium did not affect the
amplitude of the PRC even though lithium delayed the time of activity onset
during entraining conditions and increased circadian period during constant
darkness. Potassium supplementation alone, when compared to the standard diet,
increased the amplitude of the advance region of the PRC and advanced activity
phase. This study shows that phase delays due to chronic lithium are not
necessarily associated with alterations in the PRC. Dietary potassium may
influence the phase of the activity-rest cycle by acting on the advance portion
of the PRC.

INTRODUCTION

In many species, including plants, invertebrates, and mammals, lithium delays the
phase of synchronized circadian rhythms and lengthens the period of free-running
rhythms (Engelmann, 1987). Potassium intake also appears to affect circadian
rhythmicity, since a diet containing supplemental K^+ advances the phase of
activity onset in entrained Syrian hamsters (Klemfuss and Kripke, 1989). It has
been suggested that lithium alters the responsiveness of circadian oscillators to
light stimulation in mammals, possibly by acting on the retina (Carney et al.,
1988; Seggie et al., 1987), neuronal communication, or directly on central
circadian oscillators (Han, 1984; Engelmann, 1987; Delius et al., 1984). In the
present study, we examined whether lithium treatment alters the response to light
of the circadian oscillator(s) controlling wheel-running, as measured by the PRC.
We also compared PRCs in hamsters fed diets containing low and high
concentrations of potassium.

METHODS

Adult male Syrian Golden hamsters (Charles River; 110-120 gm) were maintained in
constant darkness except for 4 h of light (10-15 lux) per day. This short
photoperiod synchronized wheel-running rhythms without masking effects of light
on activity onset. Hamsters were randomly assigned to one of three test diets

from Teklad (Madison, WI). Since lithium can be toxic to rodents when administered in quantities sufficient to affect circadian rhythms, hamsters given lithium also received supplemental potassium (0.4% Li_2CO_3 + 6.96% KCl added to basic hamster diet; TD86546), which we have found to prevent toxicity in rodents while permitting effective serum lithium concentrations in the range of 0.6-1.0 mmol/L (Klemfuss and Kripke, 1989; 1987). High K^+ controls for lithium treatment were given diet containing the same high potassium concentration (1104 mmol K^+/kg diet; TD85191). An additional low potassium control group received diet in which supplemental KCl was replaced by polydextrose, a carbohydrate with minimal food value (171 mmol K^+/kg diet; TD86299). Food, water, and solid NaCl blocks were available at all times. These diets have been discussed previously (Klemfuss and Kripke, 1989).

After two weeks of treatment with lithium, high K^+ control, or low K^+ control, each hamster was transferred to a running wheel cage with the same diet and LD 4:20 regimen. Following one week of entrainment in the running wheels, each hamster was exposed to a single 15 min pulse of white fluorescent light of 325 $\pm$ 25 lux. Thereafter, hamsters remained in total darkness for two weeks.

The number of revolutions of the running wheel per 15 min epoch was recorded by a computer system. Daily activity onsets were identified by the first of three consecutive epochs with locomotor activity greater than the mesor following three epochs with activity levels less than the mesor. Activity onsets were used to determine phase position during the last week of the LD 4:20 regimen and also to determine free-running period during the second week of constant darkness, as previously described (Klemfuss and Kripke, 1989). Phase shifts due to 325 lux light exposure were calculated by standard procedures (Daan and Pittendrigh, 1976a). Briefly, the time of baseline-predicted activity onset on the day following the 325 lux light pulse (defined as CT 12) was subtracted from the time of activity onset, projected to the same day, from activity onsets during the second week after the light exposure. The resulting phase shift was plotted as a function of circadian time, where 24 circadian hours equals the hamster's tau in constant darkness. Effects of lithium and potassium were examined using analysis of variance followed by unpaired t-tests. Data were collected between January, 1987 and July, 1989, and treatments are balanced approximately by season.

RESULTS

Exposure to bright light pulses produced PRCs with small delay phase shifts early in the subjective night and large advance phase shifts in the late subjective night, as is typical for this species (Daan and Pittendrigh, 1976a). Lithium treatment clearly had no effect on the amplitude of either advance or delay shifts when compared to the high K^+ control (Fig. 1), although serum lithium concentrations averaged 0.68 mmol/L $\pm$ 0.03 SEM and both latency to activity onset and free-running tau were significantly increased (Table 1).

Comparing animals fed the low and high K^+ diets, it is apparent from Figure 1 that the high K^+ diet increased the amplitude of the advance portion of the PRC compared to the low K^+ diet. This difference was statistically significant at CT 20, CT 22, and CT 24. Table 1 indicates that supplemental K^+ significantly advanced the phase of activity onset by about one hour, but did not appear to alter tau.

PRC TO LIGHT
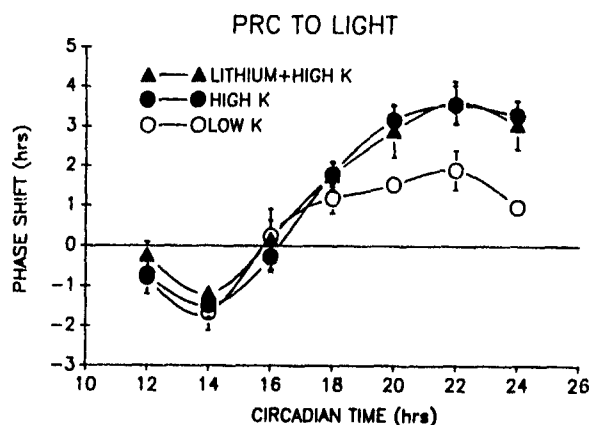
Fig. 1. Phase shifts due to 325 lux light pulse plotted against circadian time of light exposure. Mean phase shift of 5-15 hamsters/point in animals fed low K^+, high K^+, or lithium + high K^+. Standard errors larger than the diameter of data symbols are shown.

Table 1.

	Synchronized Phase (h)	Free-running Period (h)	Number of hamsters
Lithium+ High K^+	7.99 ± 0.4	24.21 ± .02	56
	]**	**[	
High K^+	5.48 ± 0.4	24.05 ± .03	50
	]*		
Low K^+	6.52 ± 0.3	24.07 ± .02	54

Table 1. Phase represents mean time between the start of darkness and activity onset, ± SEM, during an LD 4:20 schedule. Period mean ± SEM was calculated from activity onsets during the second week in constant darkness. * $p < .05$, high K^+ advances phase relative to low K^+ diet. ** $p < .001$, lithium + high K^+ delays both phase and period relative to high K^+ alone.

DISCUSSION

An effect of lithium on the light responsiveness of Syrian hamsters was reported by Han (1984). In this study, chronic lithium treatment decreased the amplitude of advance phase shifts, and slightly increased the amplitude of delay phase shifts. It is probable that differences in technique are responsible for the discrepancy between the effects of lithium in Han's PRC and our Figure 1. One

It has been suggested that animals with long free-running periods exhibit PRCs
with alterations in the relative sizes of advance and delay portions (Daan and
Pittendrigh, 1976a; Honma et al., 1985). This relationship seems to hold
(although imperfectly) when comparing species, or when comparing spontaneously
short-tau vs. spontaneously long-tau individuals, although both increases (Honma
et al., 1985) and decreases (Daan and Pittendrigh, 1976a) in the relative size of
the delay portion have been reported. Within individuals, there is evidence that
the prior light history of the animal can affect both tau and the shape of the
PRC (Daan and Pittendrigh, 1976a). However, administration of 25% deuterium
oxide (D_2O) in drinking water, which increased tau in mice by 1.8 h, failed to
affect the PRC to 15 min light pulses (Daan and Pittendrigh, 1976b). The present
results suggest that lithium, like D_2O, affects the period of central circadian
oscillators without altering light responsiveness.

Our finding that potassium treatment increased the responsiveness of the
circadian oscillator to light pulses given during the advance portion of the PRC
is consistent with the advance of the entrained activity rhythm reported here and
in our previous study using simi_ar conditions (Klemfuss and Kripke, 1989) during
potassium supplementation. Since the modest six-fold range of potassium intakes
between low and high K^+ diets is comparable to the range of daily K^+ intake in
healthy young men (National Center for Health Statistics, 1983), dietary
potassium may be useful to enhance or diminish the entraining effects of light in
people as well as hamsters.

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THE EFFECT OF CHRONIC LITHIUM TREATMENT ON RAT ACTIVITY RHYTHMS AND LEVELS UNDER CONSTANT AND ENTRAINED CONDITIONS

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ABSTRACT

Rats provided a 0.3% lithium carbonate diet developed significantly longer free-running activity rhythms during the initial sampling period after beginning treatment (Phase I-34 days) while demonstrating a nearly significant reduction in the activity/rest ratios during that same period. This classic effect was not sustained during the entire experiment. In fact, no measured circadian parameter were significantly altered after a partial crossover (Phase II-53 days) by either initiating or removing lithium treatment. During application of the LD cycle (12/12), chronically lithium treated rats did not show stable entrainment. Two distinct effects of lithium, one on a circadian pacemaker and a decrease in light sensitivity, are indicated by this study.

KEYWORDS

Lithium; Chronic; Light Sensitivity; Rat

INTRODUCTION

Lithium treatment alters circadian rhythmicity in various organisms. While many studies have associated lithium administration with longer free-running rhythms (Engelmann et al.,1976; Kripke et al., 1980) or phase delays during entrainment (Kripke et al., 1979; McEachron et al., 1982; 1985), others have reported shortened rhythms (O'Donahue et al., 1982; Subbaraj, 1981) or no effect (Christensen et al., 1982) with lithium treatment. Interactions between lithium and age, duration of treatment, or behavioral state may be able to reconcile these disparate results and provide insights into lithium's therapeutic efficacy.

MATERIALS AND METHODS

Animals and Housing

Twenty-four male rats (CD Strain), 90 days old and weighing approximately 200 grams each, were purchased from Charles River Laboratories and were individually housed in Wahmann activity cages [Lab Products, Hazelton, PA] for the duration of the experiment. These cages, which consist of a large running wheel with attached side car, were maintained in groups of six in light-tight and sound-insulated metal cabinets located in the Psychology Departments's Chronobiology Facility During light/dark (LD) cycles lighting inside the cabinets was provided by 15 watt incandescent bulbs (15 lux at the cage floor) controlled by a Chrontrol microprocessor-based timer (Lindberg Enterprises, San Diego). During constant conditions, lighting was provided by 7.5 watt red safelights (< 1 lux at the cage floor). Food (Purina Rodent Laboratory Chow 5001) and water were available ad lib throughout the experiment.Data collection was accomplished with cam-operated

microswitches connected to an IBM PC-XT-based computer analysis system (SSE Corporation, New York). Data was stored for analysis as the number of revolutions recorded each 15 minutes.

Lithium Treatment

Three diets containing lithium carbonate (at 0.075% (A), 0.15% (B), and 0.3% (C) by weight) were commercially prepared (Teklad, Madison, WI). To minimize flavor aversion, the treatment animals received diet A for the first 6 days followed by diet B for the next 7 days, followed by the maintenance diet C. All lithium-fed animals were provided with a salt (NaCl) lick to lower any toxic side effects.

Procedures

Animals were initially maintained under LD 12/12 for four weeks followed by 3 weeks in RR to insure stably entrained activity rhythms as well as sufficient activity levels for reliable estimation of rhythm parameters. One-half of the cabinets were then randomly assigned to control or lithium groups and lithium treatment initiated according to the schedule described above.

At the end of this time, selected animals of both groups were exposed to a protocol involving inescapable shock, the results of which are being published elsewhere (Stewart, et.al., in prep.).

After being fed diet C for a period of 34 days, one cabinet of six animals was randomly selected, removed from lithium treatment and provided with a regular diet until the end of the experiment (Group 1). The other animals being fed lithium were maintained as before (Group 2). At the same time, one cabinet of six animals who were previously being fed normal food began lithium treatment (Group 3). The fourth cabinet continued to receive the regular diet (Group 4). All animals were maintained under constant lighting conditions for the next 53 days at which time a 12/12 light/dark cycle (Lights on at 1900 hours EST) was imposed on all animals. This LD cycle was maintained for 35 days at which time the animals were sacrificed. Blood was collected by cardiac puncture from 11 randomly selected rats (both lithium-fed and controls) and assayed for lithium by atomic absorption spectrophotometry.

Data Analysis

Estimation of the periods of the activity rhythms involved applying least-squares cosine fits to each twenty four hours of data, followed by estimation of the best increment in degrees which would cause all acrophases to approach a single value. This value was then converted into minutes and added to 24 hours to obtain the estimated tau (McEachron et al., 1981).

The phase of each animal's activity rhythm during entrainment was also based upon application of a least-squares cosine fit with period of 24 hours to each consecutive 24 hours of data. Vector mean acrophases and standard deviations were then calculated for each animal.

The activity/rest ratios were calculated based upon the cosine estimates of tau for each animal and using the acrophase as the midpoint of activity. The ratio was calculated as the number of bins per cycle with activity present divided by the number of bins with no activity.

Tau estimates from the first day of 0.3% lithium until the day prior to the cross-over were analyzed in 2 (lithium vs. untreated) X 2 (shock vs. not shocked) ANOVAs to control for any possible effects of exposure to the inescapable shock protocol. Mean activity and activity/rest ratios were also analyzed in 2 X 2 analyses of variance.

Tau estimates from the 53 days of constant conditions after the partial crossover were analyzed in a 4 (diet history) X 2 (shock vs. non-shock) ANOVA as were activity/rest ratios and mean daily activities. Planned comparisons (Sokal and Rohlf, 1981) were run on pre- and post-crossover period estimates.

Some loss of data occurred when switches failed or individual rats produced insufficient activity to accurately estimate a period leading to variability in the degrees of freedom listed for the various statistical tests.

RESULTS

Phase I

Lithium-fed animals had significantly longer periods (Δtau of 0.274 hours) compared to rats fed a regular diet during Phase I ($F(1,19)=13.93$, $p<0.01$). This result in shown in Fig. 1.Exposing selected animals to inescapable shock, on the other hand, had no significant effect on those animals' activity periods ($F(1,19)=0.012$, ns) nor was there a significant interaction of shock and lithium ($F(1,19)=2.35$, ns). Indeed, insofar as there was never any effect or interaction linked with exposure to shock in any parameter, these results will not be discussed further.

Although not statistically significant, activity/rest ratios appeared to be reduced in the lithium-fed animals ($F(1,18)=3.50$, $p=0.077$). Mean activity levels per day were not significantly effected by lithium ($F(1,19)=2.40$,ns), shock, ($F(1,19)=0.07$,ns), or their interaction ($F(1,19)=0.988$,ns).
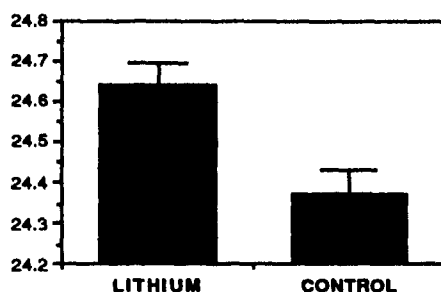
Fig. 1 Period estimates for wheel running activity in lithium-fed and control animals ($\pm$ SEM).

Phase II

Lithium had no significant effect on any measured parameter after the crossover. None of the four groups could be distinguished as significantly different in either activity periods (Fig.2) or activity/rest ratio. There were also no significant differences between the groups in daily activity levels.

Examination of the individual groups did not reveal a consistent pattern. While animals switched off lithium displayed shorter periods (a mean reduction of 0.25 ± 0.07 (SD) hours), those rats maintained on lithium in Phase II also displayed shorter periods (mean reduction of 0.13 ± 0.28 (SD) hours). Animals switched onto lithium displayed only a 0.03 ± 0.29 (SD) hour increase in period during Phase II while rats maintained on regular food the entire time showed a 0.13 ± 0.13 (SD) hour increase during this time. Planned comparisons confirm that the activity periods of rats taken off lithium could not be significantly distinguished from those animals continuously maintained on lithium ($F(1,16)=0.29$, ns) while rats exposed to lithium beginning in Phase II developed periods which were not significantly different from animals fed untreated Rat Chow for all experimental Phases ($F(1,16)=0.023$, ns).
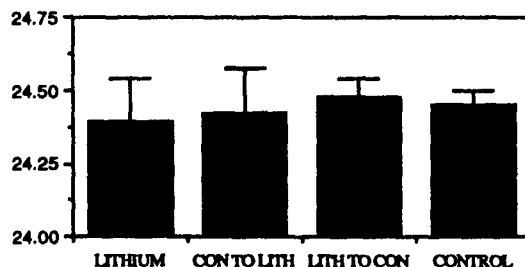
Fig. 2. Periods estimates after the partial crossover ($\pm$ SEM).

Phase III

The phase angle stability of the lithium and non-lithium groups during the LD cycle was assessed using the Scheffe F-test. The mean standard deviation of the lithium-fed animals (around each animal's mean acrophase) was found to be significantly greater (61o vs. 25o). The variance of the lithium-fed animals around the group's mean estimated period was significantly greater than animals on the regular diet (0.24 hours vs. 0.005 hours; Fmax (2,9) = 48, p<0.01). Only 2 of 9 lithium-fed animals where reliable periods were obtained during Phase III showed a period of exactly 24 hours while 6 of 10 rats being fed regular Chow displayed exactly 24 hour periods. Given recent studies linking lithium with decreased light sensitivity in bipolar patients (Carney et al., 1988; 1989), the simplest explanation for lithium-fed animals' lack of stable entrainment is a lithium-induced decrease in light sensitivity, decreasing the strength of the LD cycle as a Zeitgeber.

Mean serum lithium in rats on lithium diets was 1.24 ± 0.33 Meq/L.
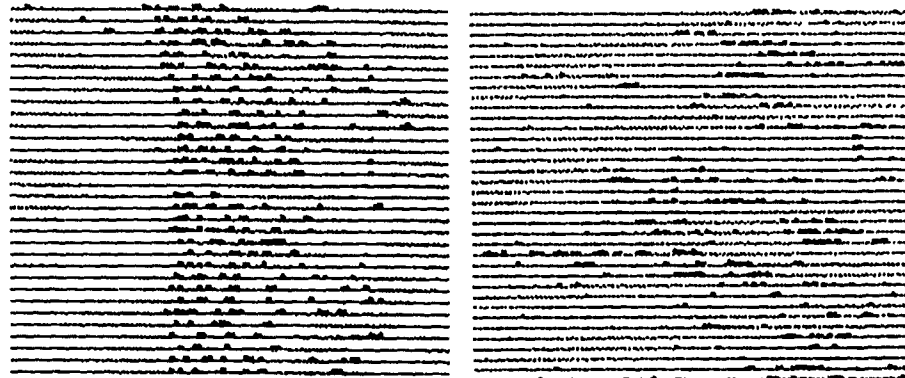
Fig.3. Actograms of rats receiving lithium (right) and regular diet (left) during exposure to LD 12/12.

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EFFECTS OF THE THIAZOLIDINEDIONE DERIVATIVE CGP 19984 ON CIRCADIAN VARIATIONS OF URINARY EXCRETION OF PINEAL AND ADRENAL HORMONES IN MALE RATS

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ABSTRACT

CGP 19984, a derivative of thiazolidinedione, has been shown to impair pituitary-gonadal activity and to suppress hormone-dependent tumor growth in rats. Since similar effects may also be elicited by the major pineal hormone melatonin, the influence of CGP 19984 on pineal activity was studied. In addition, an investigation of its influence on adrenocortical steroid production was also included. Circadian variations in adrenocortical and pineal activity were monitored in rats by determining the excretion of corticosterone, aldosterone and the major melatonin metabolite 6-sulphatoxymelatonin (aMT6s) in urine. CGP 19984 was given to male rats in oral doses of 100 mg/kg once daily on 4 consecutive days. No significant effects were found on the circadian variations of adrenocortical and pineal activity. However, urine output itself was drastically increased during the dark period in rats treated with CGP 19984 as compared to controls. In conclusion, CGP 19984 has no significant effect on pineal and adrenocortical activity. Thus, the effects of CGP 19984 on tumor growth and on gonadotropin secretion do not seem to be mediated by changes in pineal melatonin production.

KEY WORDS

CGP 19984, 6-sulphatoxymelatonin, corticosterone, aldosterone.

INTRODUCTION

The thiazolidinedione derivative CGP 19984 suppresses the growth of hormone-dependent DMBA-induced mammary tumors (Schieweck et al., 1984, and of R3327 Dunning prostate adenocarcinomas in rats (Ip et al., 1986). CGP 19984 has further been shown to reduce rat gonadotropin and subsequently gonadal steroid secretion as well as to attenuate accessory sex organ growth (Ip et al., 1986). Very similar effects can also be elicited by the major pineal hormone melatonin through effects on hormone-dependent tumor growth (Blask, 1984) and, under certain conditions, on the hypothalamo-pituitary-gonadal axis (Lang et al., 1984). Therefore, the question arose as to whether the effects observed with CGP 19984 are modulated by influencing pineal melatonin production. The present experiment was primarily designed to elucidate the effects of CGP 19984 on pineal melatonin production. Since the

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effects of this compound on the adrenal gland have not been well documented so far, an investigation of its influence on adrenocortical steroid production was also included. Circadian variations of adreno-cortical and pineal activity were monitored in sequentially collected urine by determining corticosterone, aldosterone and 6-sulphatoxymela-tonin, the major urinary metabolite of melatonin (Kopin et al., 1961).

MATERIALS AND METHODS

Male rats weighing ~200 g were kept under a lighting schedule of 14 h light:10 h darkness (lights on 06.00-20.00 h). Oral doses of 100 mg/kg CGP 19984 D, the ethyl ammonium salt of CGP 19984, were given once daily at 08.00 h on 4 consecutive days. Immediately after the third application, the animals were placed in individual metabolic cages and urine was collected by a previuosly described method (Häusler et al., 1985) from the beginning of the following dark period at 4-h intervals over a period of 36 h. The total amount of urine collected in each sampling period was determined and urine samples were stored at 20°C. The animals were weighed on day 1 and 5 of the experiment. Food and water consumption as well as urine and total hormone or metabolite excretion in the urine were measured over a 24 h-period following the fourth application. Urinary aMT6s was measured by radioimmunoassay (Aldhous et al., 1988). Unconjugated cortico-sterone in the urine was measured by a direct radioimmunoassay (Häusler et al., 1985) and aldosterone was extracted from the urine with ethyl acetate and measured by a specific radioimmunoassay. Log-transformed data for total urine and hormone output per interval were analyzed using a repeated-measures analysis of variance as described earlier (Häusler et al., 1985).

RESULTS

The data are presented in Table 1 and Fig. 1. On day 5, rats treated with CGP 19984 weighed less (P < 0.05) than the controls and 24-h food consumption of these animals was reduced (P < 0.05). Urine output was significantly increased (P < 0.01) during the dark period in the treated rats. This increased urine output paralleled the enhanced water consumption (P < 0.01) of these animals. However, there were no significant differences in the circadian pattern of urinary excretion of aMT6s, corticosterone and aldosterone between treated and control rats, although 24-h excretion of corticosterone was higher in

Table 1. Effect of CGP 19984 (100 mg/kg) given orally on 4 consecutive days on the parameters listed below (mean ± SEM).

Parameter	Unit	Time	Control (n = 7)	CGP 19984 (n = 7)
Body weight	g	Day 1	201 ± 3	208 ± 2
	g	Day 5	229 ± 6	211 ± 5*
Weight gain	g/4 days	Day 1-5	28 ± 3	3 ± 4**
Food consumption	g/24 h	Day 4-5	58 ± 4	44 ± 4*
Water consumption	ml/24 h	Day 4-5	11 ± 2	29 ± 3**
Urine volume	ml/24 h	Day 4-5	10 ± 2	25 ± 3**
aMT6s in urine	ng/24 h	Day 4-5	297 ± 28	268 ± 37
Corticosterone in urine	µg/24 h	Day 4-5	0.7 ± 0.1	1.1 ± 0.1**
Aldosterone in urine	ng/24 h	Day 4-5	6.2 ± 0.5	5.3 ± 0.6

* P < 0.05, ** P < 0.01 (CGP 19984 vs. Control, Student's t-test)
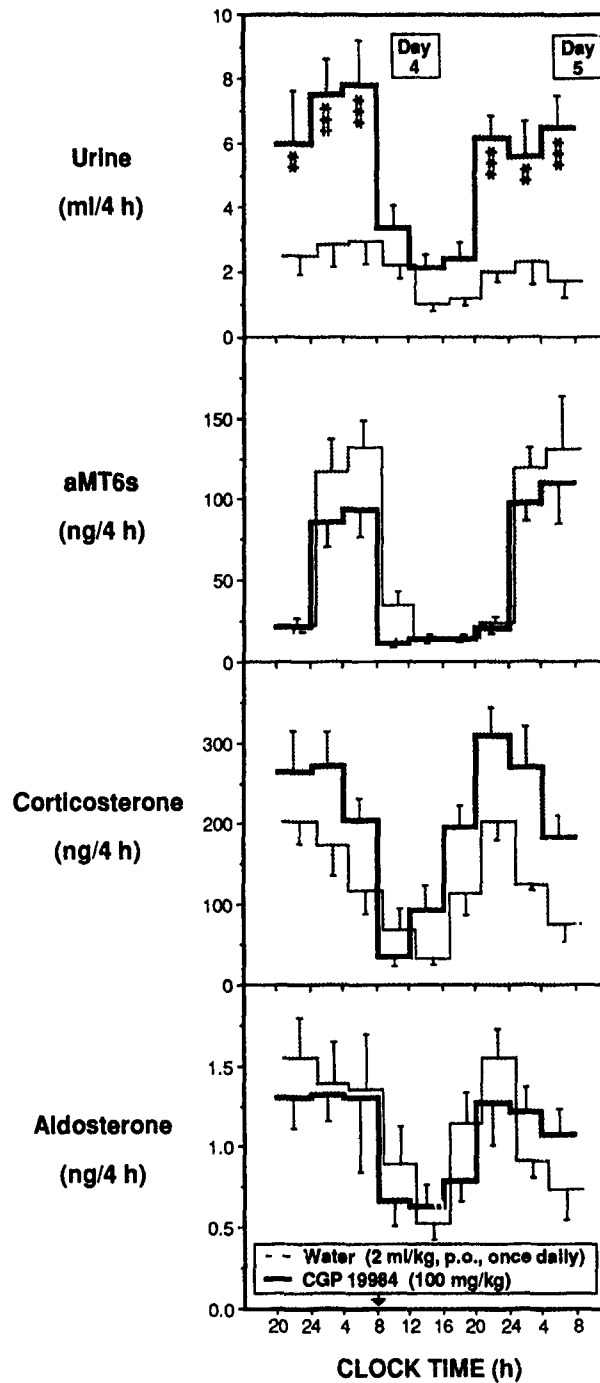
Fig. 1. Effect of CGP 19984 (100 mg/kg, p.o., once daily at 08:00 h for 4 days)
on mean (± SEM) circadian variations of urine output and of aMT6s, corticosterone
and aldosterone excretion in urine over 36 hours (** P<0.01, *** P<0.001) .
(N.B. For graphical clarity the control curve is displaced slightly to the right)

CGP 19984-treated animals than in controls. aMT6s showed a square-wave
like excretion pattern with high excretion rates between 00.00-08.00 h
and low excretion rates in-between. Both corticosterone and aldo-
sterone showed a sinusoidal excretion pattern with maximal excretion
rates in general during the first 4-8 hours of the dark period.

DISCUSSION

The reduced increase in body weight with CGP 19984 treatment confirmed
earlier findings (Schieweck et al., 1983, Ip et al., 1986) and
corresponded to the reduced food consumption in these animals. Based
on reports in the literature (Pariza, 1986) it cannot be excluded that
this (centrally mediated?) reduced food consumption contributes to the
anti-tumor effect of CGP 19984. An unexpected difference was found in
the urinary output pattern of CGP 19984-treated rats with high output
during the dark period. This increased urine output paralleled the
enhanced water consumption of these animals. The mechanism of both the
reduced food consumption and the dipsogenic effect remains unclear.
Although the accumulated excretion of corticosterone in the urine
during 24 h was significantly increased in CGP 19984-treated animals,
no effect was observed on the circadian pattern of corticosterone and
aldosterone excretion in the urine. Thus it appears that CGP 19984 has
no major effect on adrenocortical activity. Pineal activity was moni-
tored in the urine by measuring the major melatonin metabolite aMT6s
which serves as a valid approximation of melatonin produced by the
pineal gland (Markey and Buell, 1982). Typically, urinary aMT6s
excretion in the rat during the dark period was about 4-5 times higher
than during the light period as reported recently by Kennaway et al.
(1989). The circadian pattern of urinary aMT6s excretion remained
unaffected by treatment with CGP 19984. Thus based on the results
presented here, the effects of CGP 19984 on tumor growth and on
gonadotropin secretion do not seem to be mediated by changes in pineal
melatonin production.

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DAY–NIGHT DIFFERENCES IN PINEAL TYPE II THYROXINE 5'-DEIODINASE AND *N*-ACETYLTRANSFERASE ACTIVITIES DURING DEVELOPMENT IN THE RAT

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ABSTRACT

Day-night differences in type II thyroxine 5'-deiodinase (5'-D) and N-acetyltransferase (NAT) activities have been studied in the rat pineal gland during development. Day-night differences in both 5'-D and NAT activities gradually increased from 1 week of age until 6 weeks, but in a different way. Thus, NAT activity exhibited a significant nocturnal increase since the first week of life (7-fold higher than diurnal basal level), increasing gradually during development, and reaching at 6 weeks old rats night values 30-fold greater than day values. However, for pineal 5'-D activity, day-night differences were first detectable only at 2 weeks of age (2.8-fold greater than day value), increasing this difference till the last week studied (7.1-fold higher than basal level).

INTRODUCTION

During the last few years, the presence of type II 5'-deiodinase (5'-D) activity was identified in pineal gland (Tanaka et al, 1986; Guerrero et al, 1988a). The mechanisms involved in regulation of pineal 5'-D activity have been largely studied (Reiter et al, 1988; Guerrero et al, 1989). In rat pineal gland, besides the thyroid status, 5'-D activity is also regulated by the light:dark cycle, exhibiting a progressive rise in activity after the onset of the dark period and reaching a peak value 5-6 h later. This peak coincides with the nocturnal peak described for both melatonin content and N-acetyltransferase (NAT) activity (Guerrero et al, 1988a,b; Tanaka et al, 1987). This nocturnal increase in 5'-D activity seems to be dependent on the sympathetic noradrenergic input since either continuous light exposure or superior cervical ganglionectomy prevents it (Guerrero et al, 1988a,c; Murakami et al, 1988). Additionally, both in vivo and in vitro studies have shown that isoproterenol, a ß-adrenergic agonist, also activates 5'-D activity, while propranolol, a ß-adrenergic blocker, inhibits it (Guerrero et al, 1988a,d,e). No data concerning the regulation of pineal 5'-D activity during development are available. In the present work, the ontogeny of 5'-D activity in rat pineal gland is studied, and compared to the ontogeny of NAT activity. Our results indicate that nocturnal increases in both 5'-D and NAT activities can be clearly identified since one week of life.

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MATERIAL AND METHODS

Wistar rats of both sexes born in our animal facility were used. Animals received food and water ad libitum and were exposed to an automatically regulated light-dark (LD) cycle of 14:10; the lights were turned off daily from 20.00 through 06.00 h. Unless otherwise stated, rats were killed at the indicated ages and times, and pineals were quickly collected, frozen on solid CO_2, and then stored at -70 C until used.

All reagents were of analytical grade and obtained from commercial sources. T_3, D,L-dithiothreitol (DTT), Dowex-50W, tryptamine, N-acetyl-5-hydroxytryptamine, and acetyl coenzyme A were purchased from Sigma (St. Louis, MO, USA); [^{14}C]acetyl-coenzyme A and $Na^{125}I$ were purchased from Amersham (Amersham, UK). ^{125}I was bound to T_3 using the chloramine T method, as described by Nakamura et al (1977). [$3',5'-^{125}I$]T_4 was purified through a 3-ml Sephadex LH-20 column, containing the purified tracer less than 2% free iodine, and being immediately used for 5'-D analyses.

Type II 5'-D and NAT activities were determined in the same pineal. Pineals were disrupted by ultrasound in 100 µl of cold 0.05 M phosphate buffer, pH 6.8. Then, 50 and 10 µl were immediately used for determination of 5'-D and NAT, respectively. The measurement of 5'-D activity was based on the release of radioiodine from [$3',5'-^{125}I$]T_4. This method, commonly used in pineal gland studies (Tanaka et al, 1986,1987; Guerrero et al, 1988a,b,c,d,e), is sensitive enough for pineal 5'-D determinations and is specific for the type II isoenzyme, since the substrate contains ^{125}I only in position 5'. Other deiodinating activities, i.e., conversion of T_4 to rT_3, would release only nonradioactive iodide (Tanaka et al, 1986). 5'-D activity is referred to as femtomoles ^{125}I released per gland per hour. NAT activity was determined using the procedure outlined by Champney et al (1984) and expressed as either nmol N-acetyltryptamine produced per gland per hour. Results are expressed as means ± standard errors (SE). Significant differences between groups were determined by the Student's t test.

RESULTS

The day of the experiment, 1, 2, 3, 4, or 6 weeks old rats, were maintained under two different photoperiod regimens: some animals were maintained under the normal 14:10 LD cycle, with lights off at 20.00 h, and some other animals were exposed to continuous light instead of entering into the normal dark period. All animals were killed at 02.00 h (6 h after lights off), when it is described that rat pineal 5'-D and NAT activities reach maximal values (Guerrero et al, 1988b). Then, pineals were collected for determination of 5'-D and NAT activities.

As shown in Table 1, NAT activity exhibited a significant nocturnal increase since the first week of life (7-fold higher than diurnal basal level). The day-night difference increased gradually during development; thus, in 6 weeks old rats, night values were found to be 30-fold greater than day values. However, for pineal 5'-D activity (Fig. 1 and Table 1), day-night differences were first detectable only at 2 weeks of age (2.8-fold greater than day value), increasing this difference till the last week studied (7.1-fold higher than basal level).

DISCUSSION

Maturation of pineal rhythmicity has been studied in rodents, mostly rats and hamsters, and humans. These studies included the ontogeny of NAT, HIOMT, and melatonin production (Ellison et al, 1972; Klein and Stephen, 1969; Attanasio et al, 1986). The aim of this paper is to study the ontogeny of NAT and 5'-D activities determined in the same pineal gland. Our results show a parallelism in the pattern of pineal 5'-D and NAT development. Although day-night differences in 5'-D activity seem to be delayed for a week in relation to NAT, nocturnal values of both enzymes gradually increased until six weeks of age. This parallelism can suggest a possible

relationship between both enzymes in addition to a commun regulatory mechanism. Although a relationship between 5'-D and NAT activities have not been shown yet, perhaps during development this relationship can be evident.

TABLE 1. Nocturnal increases in pineal NAT and 5'-D activities during ·' velopment

	Age (weeks)				
	1	2	3	4	6
NAT activity (nmol/gland/h)					
Darkness	1.25	2.23	2.42	3,77	4.80
LL	0.18	0.14	0.15	0.15	0.16
(D/LL)*	6.94	15.92	16.13	25.13	30.00
5'-D activity (fmol/gland/h)					
Darkness	4.20	9.72	16.41	18.35	21.50
LL	3.65	3.50	3.08	2.65	3.00
(D/LL)*	1.15	2.77	5.32	6.92	7.16

*Values are referred to as the ratio between nocturnal and continuous light exposure enzyme activities.
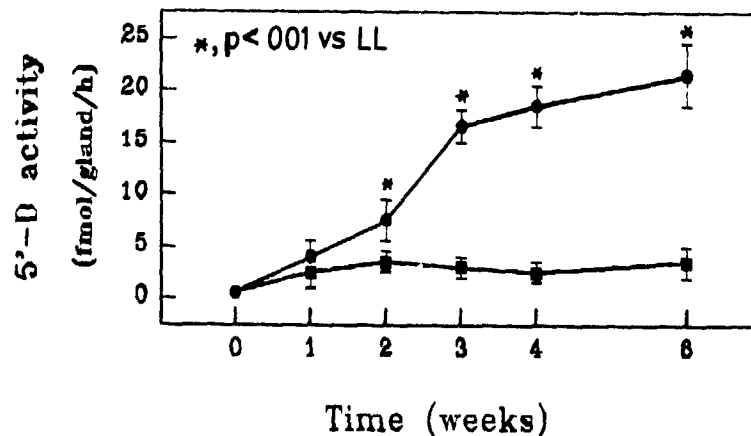
Fig 1. Pineal 5'-D activity during development. The day of the experiment, animals at indicated ages were maintained under the normal daily 14:10 LD cycle (●--●) or under LL (■--■). Then, animals were killed at 02.00 h and pineals were collected for enzyme determinations. Values are expressed as the mean ± SE of eight animals.

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NYCTOHEMERAL PROFILE OF TYPE II THYROXINE 5'-DEIODINASE ACTIVITY IN THE PINEAL AND HARDERIAN GLANDS OF THE SWISS MOUSE

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ABSTRACT

Type II thyroxine 5'-deiodinase activity in both pineal and Harderian glands of the swiss mouse were studied. We show that pineal 5'-deiodinase activity exhibits a nyctohemeral profile with a maximal peak value at 05.00 h coincident with that for pineal melatonin. However, no rhythm of 5'-deiodinase activity was found in the Harderian gland. These results suggest at least a commun regulatory mechanism for type II thyroxine 5'-deiodinase and melatonin production, and probably, an interrelationship between these two metabolic pathways.

INTRODUCTION

Rhythms in type II thyroxine 5'-deiodinase (5'-D) activity have been previously described in both pineal (Tanaka et al, 1986; Guerrero et al, 1988a) and Harderian gland (Guerrero et al, 1987) of the rat, where rhythms in melatonin production also have been described (Reiter et al, 1983; Reiter, 1986). It is believed to have an important role in some tissues in maintaining the intracellular levels of T_3 serving as a defense against thyroid hormone deficiency. The most important regulatory mechanism for this isoenzyme is the thyroid status, exhibiting an important increase in its activity during hypothyroidism and a marked inhibition in the presence of T_4 (Silva and Leonard, 1985). In rat pineal gland, besides the thyroid status, 5'-D activity is also regulated by the light:dark cycle (Reiter et al, 1988), exhibiting a progressive rise in activity after the onset of the dark period and reaching a peak value 5-6 h later; this peak coincides with the peak values described for both melatonin content and N-acetyltransferase activity (Guerrero et al, 1988a,b; Tanaka et al, 1987). This nocturnal increase in 5'-D activity seems to be dependent on the sympathetic noradrenergic input since either continuous light exposure or superior cervical ganglionectomy prevents it (Guerrero et al, 1988a; Murakami et al, 1988). In the present paper we identify, for the first time, a rhythm in the mouse pineal 5'-D activity coincident with that of melatonin. This study was performed trying to find out a new model different to rat pineal gland where to study the role of pineal type II 5'-D activity.

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MATERIALS AND METHODS

Male Swiss mice born in our animal facility were used. Animals received food and water ad libitum and were exposed to an automatically regulated light-dark (LD) cycle of 14:10; the lights were turned off daily from 20.00 through 06.00 h. In order to increase the 5'-D activity, animals were rendered hypothyroid by adding 20 mg% methimazole to the drinking water; this treatment was maintained for at least 1 week before the animals were used for the experiments. On the day of the experiments, animals were killed by decapitation at the indicated times, and pineals and Harderian glands were quickly collected, frozen on solid CO_2, and stored at - 70 C until assayed for 5'-D activity.

All reagents were of analytical grade and obtained from commercial sources. $Na^{125}I$ was purchased from Amersham (Amersham, UK). ^{125}I was bound to T3 using the chloramine T method, as described by Nakamura et al (1977).

Pineals were disrupted by ultrasound in 100 μl of cold 0.05 M phosphate buffer, pH 6.8. Then, 50 and 10 μl were immediately used for determination of 5'-D activity and melatonin content, respectively. The measurement of 5'-D activity was based on the release of radioiodine from $[3',5'-^{125}I]T4$. This method, commonly used in pineal gland studies (Tanaka et al, 1986,1987; Guerrero et al, 1988a,b,c,d,e), is sensitive enough for pineal 5'-D determinations and is specific for the type II isoenzyme, since the substrate contains ^{125}I only in position 5'. Other deiodinating activities, i.e., conversion of T4 to rT3, would release only nonradioactive iodide (Tanaka et al, 1986). 5'-D activity is referred to as femtomoles ^{125}I released either per gland per hour or per mg of protein per hour for pineal and Harderian gland, respectively. Melatonin content in the mouse pineal gland was determined after extracting with chloroform, by a commercial kit (Euro-Diagnostic BV, Apeldoorn, Holland), and was referred to as pmoles per gland. Results are expressed as means ± standard errors (SE). Significant differences between groups were determined by Student's t test.

RESULTS

The 24-h profiles of 5'-D activity and melatonin production in the swiss mouse pineal gland were studied. As shown in Fig. 1, both parameters exhibit a nyctohemeral profile with maximal peak values late in the dark period (05.00 h). During the day, values of 5'-D activity and melatonin gradually decrease until reaching minimum values before lights off and during the first third of the dark period. Then, both values raise for approximately six hours until achieving a peak at 05.00 h. Nocturnal peaks of both 5'-D activity and melatonin production elevated about four times above the basal values. During the same experiment, Harderian glands were also collected for 5'-D determinations, and Fig. 2 shows the 24-hour profile of this enzyme activity. As it can be seen, Harderian gland 5'-D activity exhibits only basal values in all time points studied.

DISCUSSION

The aim of this work is to describe the 24-hours profile of the pineal 5'-D activity in the swiss mouse. Swiss mouse is the second rodent, in addition to rat, where a pineal rhythm in 5'-D activity has been found. Neither Syrian hamster nor Richardson's ground squirrels have been found to exhibit a this rhythm (Puig-Domingo et al, 1988). Moreover, the enzyme exhibits a nocturnal maximal activity at 05.00 h, which is the time when pineal melatonin content also reaches maximal value. Similar coincident profiles in pineal 5'-D activity and melatonin content have been previously described in the rat (Guerrero et al, 1988b). On the other hand, swiss mouse Harderian gland exhibits only basal values in 5'-D activity during the daily light-dark cycle. In conclusion, swiss mouse is a new model to study the pineal 5'-D activity rhythm. and its possible physiological role in the pineal metabolism.
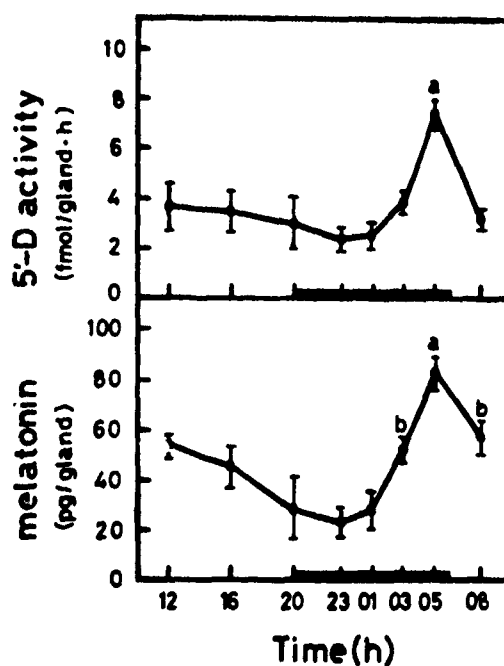
Fig. 1. Thyroxine 5'-D activity (top) and melatonin content (bottom) in the swiss mouse pineal gland. Animals were killed at the indicated times and pineals quickly collected for 5'-D activity and melatonin content determinations. Each value is the mean ± SE of eight animals. (a, $p < 0.001$ vs basal value at 23.00 h; b, $p < 0.01$ vs basal value at 23.00 h).
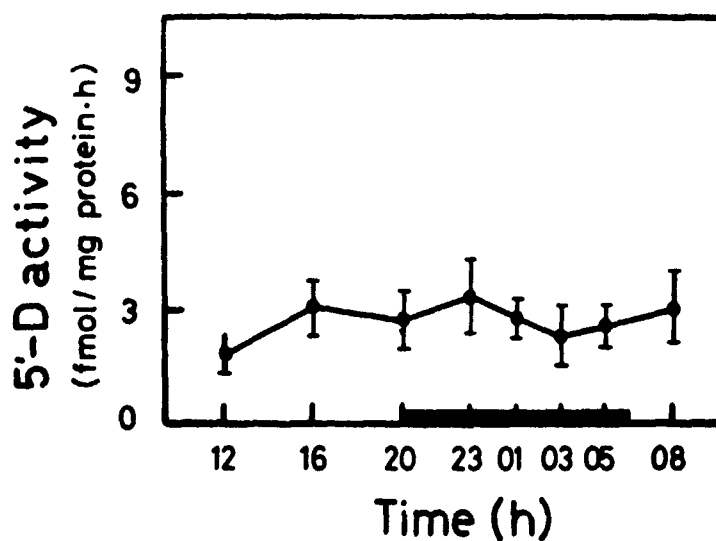
Fig. 2. Thyroxine 5'-D activity in the swiss mouse Harderian gland. Animals were killed at the indicated times and Harderian glands quickly collected for 5'-D activity determination. Each value is the mean ± SE of eight animals.

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NOREPINEPHRINE STIMULATION OF RAT PINEAL TYPE II THYROXINE 5'-DEIODINASE

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ABSTRACT

In the present paper we show, for the first time, an in vivo activation of rat pineal thyroxine 5'-deiodinase activity by injecting norepinephrine, the physiological neurotransmitter released by sympathetic nerve endings. In this work we performed the experiment injecting norepinephrine joined to DMI, which inhibits the norepinephrine uptake by the nerve terminals. Under these conditions norepinephrine exhibits a very potent effect in stimulating rat pineal thyroxine 5'-deiodinase activity. Moreover, the response to norepinephrine is greater in pineals collected from euthyroid animals, confirming that during hypothyroidism, pineal gland is supersensitive to adrenergic agonists probably due to the presence of larger quantities of mRNA for the synthesis of the enzyme.

INTRODUCTION

Rhythms in thyroxine type II 5'-deiodinase (5'-D) activity have been described in rat pineal (Tanaka et al,1986; Guerrero et al, 1988a). In rat pineal gland, besides the thyroid status, the 5'-D activity is also regulated by the light:dark cycle, exhibiting a progressive rise in activity after the onset of the dark period and reaching a peak value 5-6 h later; this peak coincides with the peak values described for both melatonin content and N-acetyltransferase activity (Guerrero et al,1988a,b; Tanaka et al,1987). This nocturnal increase in 5'-D activity seems to be dependent on the sympathetic noradrenergic input since either continuous light exposure or superior cervical ganglionectomy prevents it (Guerrero et al,1988a,c; Murakami et al, 1988). Additionally, both in vivo and in vitro studies have shown that isoproterenol, a ß-adrenergic agonist, also activates the 5'-D activity, while propranolol, a -adrenergic blocker, inhibits it (Guerrero et al, 1988c,d,e). In the present paper we activated, for the first time, the rat pineal 5'-D activity by injecting norepinephrine (NE), the physiological neurotransmitter released by the sympathetic nerve endings. This study was performed trying to define the role of α-adrenergic receptors, in addition to ß-adrenergic receptors, in stimulating pineal 5'-D activity.

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MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing approximately 100 g, were purchased from Harlan (Indianapolis, IN) and allowed to acclimate to the animal facilities. Animals received food and water ad libitum and were exposed to an automatically regulated light-dark (LD) cycle of 14:10; the lights were turned off daily from 20.00 through 06.00 h. When required, animals were rendered hypothyroid by adding 20 mg% methimazole to the drinking water; this treatment was maintained for at least 1 week before the animals were used for the experiments. On the day of the experiments, animals were killed by decapitation and pineals quickly collected, frozen on solid CO_2, and stored at -70 C until assayed for 5'-D activity.

The measurement of 5'-D activity was based on the release of radioiodine from T_4. This activity is specific for type II 5'-D, since the substrate contains ^{125}I only in position 5'. Other deiodinating activities , i.e., conversion of T_4 to rT_3, would release only nonradioactive iodide (Tanaka et al, 1987). Briefly, pineals were disrupted by ultrasound in 100 µl of cold 0.05 M phosphate buffer, pH 6.8. Then 50 µl were immediately incubated in the presence of 40 mM DTT and 2nM [3',5'-^{125}I]T_4 as substrate (200 µl final volume). The substrate concentration was similar to the K_m value described for 5'-D activity in rat pineal (Tanaka et al,1986). Reaction was started by the addition of the substrate and continued for 60 min at 37 C. Control incubations were performed by omission of the homogenates. The reaction was terminated by the addition of 100 µl of cold 2% bovine serum albumin and 750 µl of 10% trichloroacetic acid. The samples were centrifuged for 10 min at 3.000 rpm and the supernatant was decanted onto a 1.5 ml column packed with Dowex-50W ion-exchange resin and eluted with 2 ml of 10% glacial acetic acid. Radioactivity in the eluate, corresponding to the ^{125}I released, was counted in a gamma counter as an index of 5'-D activity. The recovery of ^{125}I in this process was better than 95%. Specific enzymatic activity was determined by subtracting the control value, which usually amounted to less than 1% of the radioactivity added. 5'-D activity is referred to as femtomoles of ^{125}I released per gland per hour. Results are expressed as means ± standard errors (SE). Significant differences between groups were determined by Student's t test.

All reagents were of analytical grade and obtained from commercial sources. T_3, D,L-dithiothreitol (DTT), (-)-isoproterenol (ISO), (-)-norepinephrine (NE), L-phenylephrine (PE), prazosin (PRAZ), DL-propranolol (PROP), and desmethylimipramine (DMI) were purchased from Sigma (St. Louis, MO); Na^{125}I was purchased from Amersham (Arlington Heights, IL). ^{125}I was bound to T_3 using the chloramine T method, as described in (Nakamura et al,1977), and purified through a 3-ml Sephadex LH-20 column. The purified tracer contained less than 2% free iodine and was immediately used for 5'-D analyses.

RESULTS

In this experiment the in vivo effect of NE and ISO on 5'-D activity was tested. Fifty-six rats (eight groups of seven each) were used in this study. The half of the animals were rendered hypothyroid to increase the response of rat pineal 5'-D activity to ß-adrenergic agonists (Guerrero et al,1988b). On the night of the experiment, groups of both euthyroid and hypothyroid animals were maintained under light conditions, and injected (s.c.) with either saline, ISO (1.0 mg/Kg BW), NE (1.0 mg/Kg BW) + DMI (5 mg/Kg BW)), or DMI (5 mg/Kg BW) at each of the following times, 20.00, 22.00, and 00.00 h. Then, animals were sacrificed at 01.00 h and pineals quickly collected. DMI was used to prevent the uptake of NE by the sympathetic nerve terminals. As shown in Figure 1, administering either ISO or NE plus DMI under light conditions at night, clearly increased pineal 5'-D activity in both euthyroid and hypothyroid animals. However, the response of hypothyroid animals to drugs is greater; even basal values of 5'-D activity, in the absence of drugs, are enhanced during hypothyroidism. On the other hand, the effect of NE plus DMI on 5'-D activity seems to be more potent than that exhibited by ISO.

Although the effect of NE alone has not been studied, its effect on 5'-D activity seems clear since injecting DMI alone had no effect on the enzyme activity in both euthyroid and hypothyroid animals.
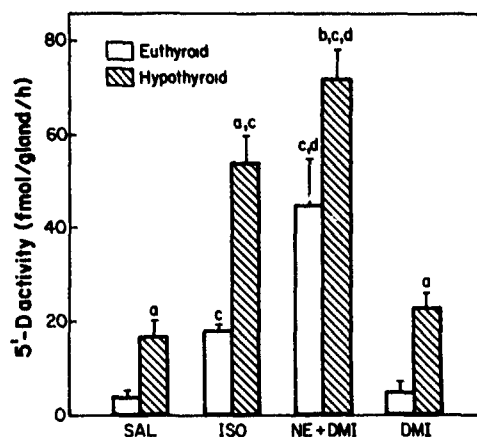
Fig. 1. Effect of either isoproterenol (ISO), norepinephrine plus desmethylimipramine (NE + DMI), or desmethylimipramine alone (DMI) on pineal 5'-D activity of euthyroid and hypothyroid rats. Rather than entering darkness at 20.00 h, rats were maintained under light and were repeatedly injected subcutaneously (20.00, 22.00 h, and 00.00 h) with either saline (SAL), ISO (1.0 mg/Kg BW), NE + DMI (5 mg/Kg BW), or DMI alone. Animals were killed at 01.00 h and pineals were collected for 5'-D activity determinations. Results are the mean ± SE of seven animals/group. (a, p < 0.001 vs euthyroid animals; b, p < 0.05 vs euthyroid animals; c, p < 0.001 vs SAL; d, p < 0.01 vs ISO).

DISCUSSION

Regulation of type II thyroxine 5'-D activity has been studied in many tissues, organs, and species. Thyroid status is both the most important and commun regulatory mechanism described for the 5'-deiodinase isoenzyme. But, besides the thyroid status, noradrenergic input also regulates type II 5'-D activity. Thus, in rat pineal gland, nocturnal noradrenergic input is responsible of increasing not only N-acetyltransferase activity and melatonin production but also 5'-D activity (Guerrero et al,1988a,b; Tanaka et al,1987; Murakami et al,1988). The primary role of ß-adrenergic receptors in the rat pineal activation of 5'-D was shown both in vivo and in vitro, since isoproterenol, a ß-adrenergic agonist, was able to stimulate 5'-D activity (Guerrero et al,1988c,d,e). Moreover, propranolol, a ß-adrenergic receptor blocker, abolished the pineal 5'-D nocturnal peak (Tanaka et al,1987; Guerrero et al,1988c). In the present paper we show, for the first time, an in vivo activation of rat pineal 5'-D activity by injecting norepinephrine, the physiological

neurotransmitter released by sympathetic nerve endings. In previous experiments (Murakami et al,1989) norepinephrine was incapable of stimulating rat pineal 5'-D activity. However, in this work we performed the experiment injecting norepinephrine joined to DMI, which inhibits the norepinephrine uptake by the nerve terminals. Under these conditions norepinephrine exhibits a very potent effect in stimulating rat pineal 5'-D activity (Fig. 1). Moreover, the response to norepinephrine is greater in pineals collected from euthyroid animals, confirming that during hypothyroidism, pineal gland is supersensitive to adrenergic agonists probably due to the presence of larger quantities of mRNA for the synthesis of the 5'-D enzyme, as suggested in (Guerrero et al,1988e). In conclussion, norepinephrine stimulates pineal 5'-D activity and its effect is more potent than that obtained with isoproterenol, suggesting that in addition to ß-adrenergic receptors, α-adrenergic receptors might be involved in the 5'-D activation.

ACKNOWLEDGMENTS

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BRIGHT LIGHT IN THE MORNING BUT NOT IN THE EVENING AFFECTS CIRCADIAN RHYTHMS IN PLASMA cAMP, MELATONIN AND CORTISOL IN HEALTHY SUBJECTS

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SUMMARY

In 18 young, healthy volunteers rhythms in plasma cAMP, melatonin (M) and cortisol (C) were studied after bright light (BL) either in the morning (5.00-8.00h) or in the evening (18.00-21.00h). In addition grip strength (GrS) was measured over the same 24h period. Blood samples were taken every 3h within 24h before (control) and after 6 days of BL. Plasma cAMP, M and C were determined by radioimmunoassay. During whole study volunteers had to live under an activity/rest cycle with nocturnal rest from 23.00-5.00h, controlled by actometers. Before BL 16 out of 18 subjects showed a significant rhythm in plasma cAMP with an acrophase at 13.54h. After 6 days of BL in the morning only 4 out of 12 volunteers showed significant rhythm in plasma cAMP. After BL in the evening 5 out of 6 subjects exhibited significant cAMP rhythms without difference in the acrophase. Mean acrophases in control rhythms of M (3.3h) and C (10.6h) were significantly advanced by BL in the morning by 1.4h and 2.2h respectivly, but not by BL in the evening. The rhythm in GrS was not altered by either BL treatment.

KEY WORDS

morning/evening bright light, plasma cAMP, melatonin, cortisol, grip strength, healthy subjects, daily rhythm.

INTRODUCTION

Circadian rhythm in plasma cAMP was demonstrated in controls and in patients with affective illness (1). Rhythmic secretion of C and the light-dependent variation of M are well known (2). BL which is effective in the treatment of patients with seasonal affective disorders (SAD; 3) is moreover responsible for a phase advance of the melatonin cycle in SAD-patients (2). Since functions of the adrenergic nervous system, the suprachiasmatic nucleus and the pineal gland are thought to be involved in SAD, we hypothesized that application of BL could affect the circadian rhythms in cAMP, M and C. Thus we investigated whether or not circadian variation of the parameters measured were influenced by BL applied either in the morning or in the evening.

MATERIAL AND METHODS

18 young, healthy volunteers were divided in two groups: 12 volunteers (7 f, 5 m, 21-27 y) with application of BL from 5.00-8.00h (Group 1), 6 volunteers (2 f, 4 m, 22-27 y) with application of BL from 18.00-21.00h (Group 2). Light intensity was at least 2500 Lux. Both groups received BL over 6 days (day 2 to 7) with 24-h-measurements on day 1 and on day 8. Blood samples for all parameters were taken every 3h over a 24h period on day 1 and 8, starting at 11.00h with repeating last sample at 11.00h next day. In order to avoid an influence of early morning rising, both groups had to live under the same activity/rest-cycle with nocturnal rest from 23.00 - 5.00h. Body activity was controlled by actometers (ZAK-Aktometer) during the whole study. Blood samples for plasma cAMP (1.9 ml) were added with 0.1 ml 2% EDTA containing 4 mM of the

	BL morning		BL evening	
	pre BL	post BL	pre BL	post BL
cAMP	13.5 ± 1.0	---- *	14.2 ± 0.9	14.7 ± 0.6
Melatonin	3.3 ± 0.2	1.9 ± 0.4*	2.1 ± 0.3	1.9 ± 0.4
Cortisol	10.6 ± 0.3	8.4 ± 0.3*	10.0 ± 1.0	10.3 ± 1.0

Tab. 1: Group mean acrophases (h) pre and post BL.
* not calculated, 8 out of 12 not significant. * $p < 0.01$

phosphodiesterase-inhibitor isobuthylmethylxanthine (IBMX). Samples for M and C were taken at the same time-points. Grip strenght was determined by dynamometers (Colin Gentile, Paris) at least every 3h on day 1 and 8. Concentrations of plasma cAMP, M and C were determined by radioimmunoassay. Data of each single volunteer were fitted to a cosine function by a computerized fitting routine (4). Acrophase, amplitude and mesor were calculated, tau=24h. The effect of BL on circadian rhythm in C and M was statistically verified by paired t-test. For plasma cAMP and GrS data of single cosinor analyses were expressed as % of individual means. Group mean (%) was calculated at each time point and analyzed by the cosinor method. Statistical significance was tested by one-way ANOVA.

RESULTS

Before BL 16 out of 18 subjects showed a significant circadian rhythm in plasma cAMP (10 out of 12, group 1; all 6, group 2) with a mean acrophase at 13.54h. Mean plasma cAMP concentrations were subject dependent (10-22 pmol/ml). After BL only 4 out of 12 volunteers in group 1 exhibited circadian variation in plasma cAMP. In contrast still 5 out of 6 subjects in group 2 showed significant daily variation of plasma cAMP. Group mean cosinor showed significant circadian variation of plasma cAMP in group 2 , but not in group 1 (Fig. 1). Acrophase and amplitude were not affected by BL in the evening. In group 1 BL in the morning significantly ($p < 0.01$) advanced acrophase in rhythmic secretion of M by 1.4h. An advance in acrophase was present in each single subject. After BL in the evening no change in acrophase could be found for M (Fig. 2). According to these data, acrophase in C was advanced by BL in the morning (10.62h vs. 8.39h) but not in the evening (Fig. 3). GrS was not altered by either BL treatment: Group mean cosinor was highly significant in both groups before and after application of BL.
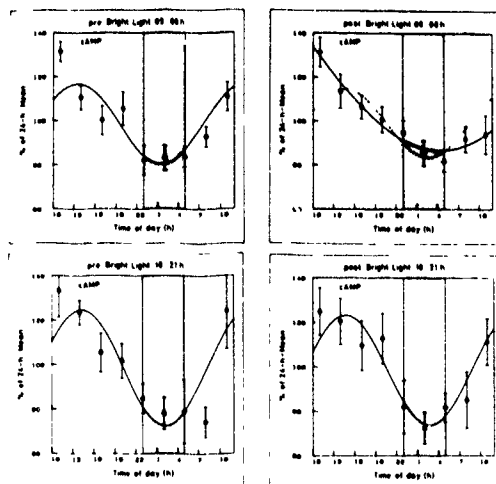
Fig. 1: Circadian variation in cAMP for BL-morning (upper part) and
BL-evening (lower part) before (left) and after (right) BL. Dotted line:
tau=24h, n.s., solid line: tau=48h, p< 0.05.
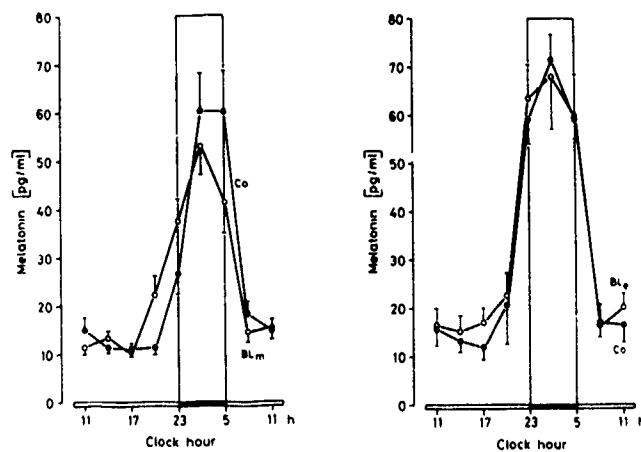
Fig. 2: Circadian rhythm in melatonin for group 1 (left) and group 2
(right) in controls (Co) and after application of BL (BLₘ vers. BL.).

DISCUSSION

In the present study circadian variation in plasma cAMP could be demonstrated either in controls or after BL treatment. The calculated acrophase (13.54h ± 0.54h) was in good agreement with earlier findings (1,5). Lemmer (5) reported an acrophase in plasma cAMP of 12.20h ± 1.1h in healthy volunteers who got up at 7.00h, indicating that the activity/rest cycle per se is not responsible for rhythmicity in plasma cAMP. Early-morning BL induces phace advance of M-cycle in SAD patients (6). The present study demonstrates the same effect of BL in healthy subjects. In addition, phase advance in cortisol rhythmicity could be shown after BL in the morning but not in the evening (Tab. 1). Since both groups had to live under the same activity/rest cycle with standing up in the early morning it is assumed that BL in the morning is responsible for abolition
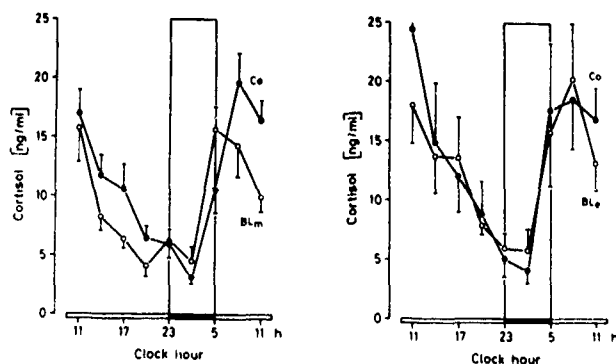
Fig. 3: Daily rhythm in cortisol for group 1 (left) and group 2 (right) in controls (Co) and after application of BL_m and BL_o.

of the rhythmicity in plasma cAMP in most subjects and for the significant phase advance in the rhythms in M and C, whereas BL in the evening was without effect on either parameter.

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BRIGHT LIGHT AMELIORATION OF SHIFT WORK

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ABSTRACT

Five groups (four treatment and one control) of military volunteers were trained to perform a computerized task battery and then worked a full day shift, followed by an all night shift before sleeping during the day. After the sleep period the men worked another night shift. Each group except the control group received a dose of bright light (2000 lux) for three hours at different times during the first night shift in an attempt to suppress melatonin. Samples of blood and measures of core temperature, activity, illumination and performance were taken repeatedly. As of this writing we have studied 22 of the 50 subjects scheduled. Preliminary analysis indicates equal or better results across all groups on the second night shift as compared to the first night for Simple Reaction Time, Logical Reasoning, Addition/Subtraction, Sleepiness and Fatigue.

KEYWORDS

Circadian Rhythms, Shift Work, Melatonin, Bright Light, Sustained Operations

INTRODUCTION

Studies of night shift workers (Kripke, 1989) and workers in continuous operations (Englund, et al, 1985) demonstrate impaired performance, drowsiness, fatigue, impaired mood and decreased sleep. Current theory predicts that bright light treatments should help night shift workers, both by shifting circadian rhythms and by suppressing melatonin, a nocturnally-secreted hormone which is associated with sleepiness, fatigue and perhaps decreased performance (Weaver, et al, 1983; Czeisler and Allan, 1987). Our goal has been to explore applications of bright light in a military shift work model. To guide the development of safe and effective bright light treatments, we are examining the effects of bright light on mood, activity, performance, and circadian rhythms.

MATERIALS AND METHODS

The research design contrasts performance in bright light-treated groups with comparable performance in parallel dim-light-treated control groups. Four treatment and one control group of ten subjects each will be contrasted to determine optimum light treatment modalities. Typically two subjects are studied each week. Subjects are trained on computerized tasks selected from a performance assessment battery (Englund, et al, 1987) during their first full

day in the laboratory to establish baseline performance levels. The first sleep period is from 2200-0600. Continuous work begins at 0800 wednesday until 1600. Work starts again ﹖t 1900 wednesday until 0700 thursday. A sleep period is given at 0800-1600 thursday, wherein another continuous work segment starts at 1900 through 0700 friday. Bright light (2000 lux) is administered for three hours during the first continuous night-work segment at 1900, 2200, 0100 or 0400, depending upon which group a subject is assigned. The controls receive a dim red light placebo during 0100-0400. Except for the bright light treatment period, all continuous work is conducted under 200 lux ambient lighting. Core body temperature, wrist activity, and illumination exposure are monitored by a Vitalog PMS-8 attached to the subject throughtout the study (Kripke and Gregg, 1990; Kripke, et al, 1981). Cognitive performance and mood are assessed by subjects working continuously at a computer which presents tasks and profiles in the form of a performance assessment battery. The task battery is repeated once each hour, permitting five to ten minute breaks every half hour during which time blood samples are obtained. Six cc samples of blood are drawn every 30 minutes from 1900-0700, which is expected to include the entire nocturnal melatonin elevation.

The Performance Assessment Battery is a subset of the Unified Tri-service Cognitive Performance Assessment Battery. Performance tasks include alpha-numeric visual vigilance monitoring, four choice reaction time logical reasoning, simple reaction time, and spatial processing. Specific measures of mood include the Profile of Mood States, the School of Aerospace Medicine Subjective Fatigue Check list, and Stanford Sleepiness Scale.

The Vitalog PMS-8 is a commercial "Walkman"- sized monitoring computer which subjects wear on a belt. Kripke and colleagues have developed software and transducers to monitor wrist activity, core temperature, and illumination exposure. Activity is measured with calibrated precision accelerometers. It has been shown that over intervals beyond 10 seconds, single axis acceleration correlates very highly with multi-axial acceleration. Illumination is measured with a photocell placed under a green glass filter to approximately estimate photopic illumination with a cosine-corrected field of view, covering a calibrated range of 1-50,000 lux. For log-lux, the correlation with these transducers between wrist and forehead placements over the eyes is excellent (r=0.90). Core temperature is measured with FDA approved sterile disposible Yellow-springs rectal thermistors.

RESULTS

French and colleagues (1990) found significant oral temperature increases and improved response time scores with reduced errors in bright-light treated males working all night. Subjects in that study experienced doses of 3000 lux for the entire work episode lasting for 14 hours. Thus far, 22 subjects have completed the current arduous study which is still in process. A first level analysis of the data yields a tendency for differences (however not significant) between groups (due to limited cell size, individual differences, and perhaps the treatment procedure), and modest but significant differences between work episodes (day work, first night shift and second night shift). These effects are most noticeable for measures of fatigue, and addition-subtraction, logical reasoning and simple reaction time tasks (see figure 1). The improvement of scores on the second night are in the anticipated direction of change . An analysis of individual profiles of mood such as sleepiness and task responsiveness also supports this tentative conclusion. The subjects have indicated less sleepiness, and some subjects verbally report that light treatment has been helpful. Given the limited sample size, this modest response to light is interesting in view of the lower dose level given for the shorter time as compared to studies using higher doses for longer periods.

SIMPLE REACTION TIME, LOGICAL REASONING, AND ADDITION/SUBTRACTION Z-SCORES

TIME OF DAY

POMS (FATIGUE SCALE)

TIME OF DAY
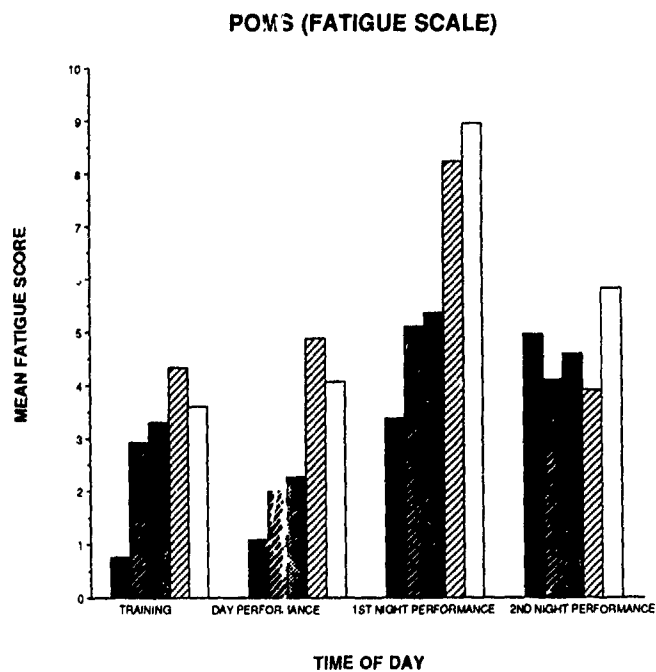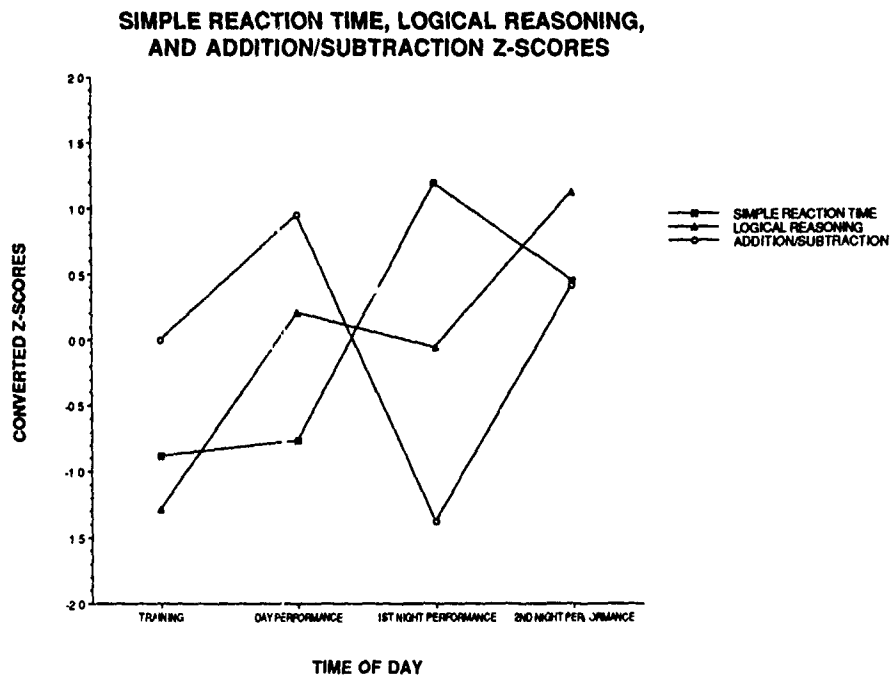
Fig 1   Mood and performance changes after treatment with bright-light.

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EFFECTS OF BRIGHT ILLUMINANCE ON BODY TEMPERATURE AND HUMAN PERFORMANCE*

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ABSTRACT

Bright (3000 lux) vs dim (100 lux) illuminance levels were compared for as-
sociated effects on oral temperature and sustained human performance. A counter
balanced repeated measures design was used to assess the 9 male subjects during
each of the two illuminance conditions. After practice sessions (0700 - 1800
hrs), cognitive performance tests were administered by a computer workstation
every 2 hrs throughout the test period (1800 - 1000 hrs). Oral temperature
taken immediately after each performance test was elevated during the bright
over the dim condition at 2130, 0130, 0330 hrs. Performance on tests for
cognitive abilities was improved for the bright over the dim light condition
particularly at 2400, 0200 and 0400 hrs. This effect did not endure after the
bright light exposure ended. These data suggest that bright illumination may
improve performance otherwise susceptible to fatigue, particularly during early
morning shifts.

KEYWORDS

fatigue; illuminance; temperature; performance; light

INTRODUCTION

Many studies suggest that melatonin acts as an endogenous sleep promoting com-
pound in humans. For example, melatonin appears to have a depressant effect
upon arousal, attention and motor activity in animals (Anton-Tay et al., 1971).
Human subjects given relatively low doses (2 mg) of melatonin for three weeks
experienced increased fatigue (Arendt et al., 1984). Similarly, an acute oral
dose of 240 mg of melatonin elevated fatigue, increased confusion and slowed
reaction time (Lieberman et al., 1985). Additionally, plasma melatonin levels
are greatest during the peak of rest phase of the human rest activity cycle.

Exposing the eye to light acutely suppresses elevated nocturnal plasma melatonin
levels in rodents (Benshoff et al., 1987), monkeys (Brainard et al., 1981) and
humans (Lewy et al., 1980; Brainard et al., 1988). In addition, cortisol,
core temperature and sleep-wake cycles can be shifted in subjects when bright,

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broad spectrum illumination (2000-3000 lux) is administered at specific times during the 24 hr day (Czeisler, 1986). Similarly, the administration of 3000 lux illumination for a period of 7 hours (early evening through early morning) delayed the onset of 6-sulphatoxy melatonin excretion the following evening (Kennaway et al., 1987). Hypothesizing that elevated levels of melatonin may contribute to night time fatigue and given its attenuation or phase shifting by bright light, the current study addressed the effects of illumination intensity on reducing fatigue and improving sustained performance.

MATERIALS AND METHODS

A counter balanced within subjects ANOVA design was used to compare 10 male subjects exposed to bright (3000 lux; 951 uW/cm^2) and dim (100 lux; 30 uW/cm^2) lighting conditions. Subjects were recruited from non-smoking civilian and military personnel and were required to refrain from all drugs for 24 hours and to get a normal evening rest prior to testing. Five subjects were tested at a time and each subject was assigned to an individual testing booth that contained a wide spectrum fluorescent illumination source (Vita-Lite, Duro-test Corp., Fairfield, N.J. USA) and a PC workstation. Each booth was separated from adjacent booths by sound attenuating, frame partitions that restricted the subject's view to their workstation. A comfortable chair allowed the subject to sit close to the workstation. The light source fixture was suspended on a wooden frame over each workstation and could be adjusted to administer either the bright or dim illuminance treatment. One subject was unable to complete the study. All of the subjects signed consent forms to participate. The study protocol was approved by the Advisory Committee on Human Experimentation at the School of Aerospace Medicine.

Subjects arrived at the lab at 0700 to permit familiarization with the test battery procedures. The first 3 trials were used to stabilize performance under dim illumination and occurred between 1000, 1400 and 1600 hrs. These trials were followed by 7 treatment trials under either the bright or the dim illumination conditions and occurred every 2 hours between 1800 and 0600 hrs on the following day. Each two hour cognitive test battery sequence was followed immediately by a sublingual temperature reading. A secondary objective was to assess the duration of any illumination effects following a return to the dim condition for 2 additional trials (4 hours) between the hours of 0800 and 1200 hrs on the second day. The subjects were required to stay in the booth through-out the study with the exception of short (< 5 minute restroom breaks). Social interactions between subjects were kept to a minimum by the experimenter and by the demands of the testing schedule. There were at least 2 weeks between the first and second experimental sessions for each group to allow re-entrainment of the circadian cycle and recovery from sleep loss. The same foods were served to the subjects in their booths during both light conditions and at the same times.

The cognitive tests were taken from the Complex Cognitive Assessment Battery (CCAB) and the Walter Reed Assessment Battery (WRPAB) which were developed for use by the US military[*]. Dependent measures consisted of response time and accuracy variables. The order that the tests were presented did not vary throughout the study. The 5 performance tests used from the WRPAB consisted of the choice reaction time (CRT), column addition and subtraction (CAS), the manikin test (MT), serial addition and subtraction (SAS) and Wilkinson reaction time (WRT). Two subjective measures were also taken, an automated version of the Profile Of Mood Survey (POMS) and the Stanford Sleepiness Scale (SSS). The 5 tests used from the CCAB were the tower puzzle (TP), following directions (FD), the numbers (N) and words (W) dual process task and route planning (RP).

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RESULTS

Subjective profiles based upon the POMS and the SSS indicated the lighting conditions could not be distinguished in terms of fatigue nor any mood dynamics nor level of sleepiness. However, oral temperature levels were significantly elevated (p < .05) by the bright light compared to the dim light condition at 2130, 0130 and 0330 hrs as shown in Fig. 1.
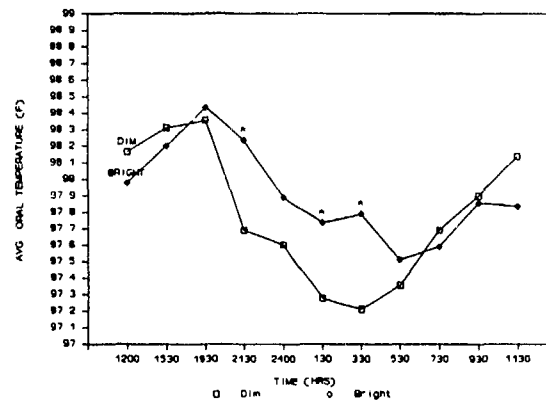
Fig. 1. Sublingual temperature across time awake comparing the bright (3000 lux) and dim (100 lux) illuminance conditions (* = p < .05).

The results of the cognitive tests are shown in Table 1. Seven of the tests (CRT, RP, SAS, N, W, FD and MT) were sensitive to the effects of the illuminance conditions. The 2400, 0200 and 0400 time points are associated with the most beneficial effects of bright light exposure as shown in Table 1. These times are similar to those in which oral temperature was elevated. However, beginning at 0600 and throughout the times in which the lights returned to dim practice conditions, subjects exposed to bright light began to perform worse (less accurately, greater response time) than during the dim condition. Light exposure seemed to have beneficial effects on SAS, RP and N tasks as shown in Table 1 whereas the FD task seemed to be the most susceptible to disruption following extended exposure to bright light. Only response time performance variables were improved for the SAS test throughout the test session while FD and W demonstrated alterations in response time and accuracy variables at the times indicated. Only accuracy variables were susceptible on the N, CRT and the RP tasks. All of these results represent light x time awake interaction effects (p < .05). The bright condition improved an accuracy variable (number of errors) on the MT as a main effect across all time points.

Bright light exposure may improve performance otherwise susceptible to fatigue. However, it appears that there is no duration of light effects beyond the exposure period. In fact, since performance begins to degrade somewhat after 10 hours of bright light exposure, the effectiveness of the lights in reducing fatigue degraded performance may have been exceeded. These results suggest that bright lights will have their greatest effect on fatigue degraded performance in the early morning hours following midnight. The disruption of performance following the bright light exposure may be related to an initially greater metabolic demand stimulated by the light as indicated by the increase in oral temperature and an improvement in performance. This may have finally resulted in a reduction of energy reserves and the eventual degradation of performance. Alternatively, if the anti-fatigue effects shown here are the result of changes in melatonin then the 0600 decrement in performance may be related to an inability of 3000 lux to continue to suppress melatonin followed by a rebound

increase in melatonin levels when light exposure terminated. Both of these
possibilities are currently under investigation.

Table 1. The number of cognitive test results either improved or made
worse by the application of bright lights at sequential times. A total of 10
performance tests were given at each time. The individual tests are identified
(parentheses) at each time point. (- response time; ⊤ accuracy; ± both af-
fected).

TIME	# OF TESTS IMPROVED	# OF TESTS WORSENED
Light on		
1800	1 (CRT+)	0
2000	1 (RP+)	0
2200	1 (SAS-)	0
2400	4 (SAS-, N+, W+, FD±)	0
0200	2 (SAS-, N+)	0
0400	2 (SAS-, N+)	0
0600	0	1 (FD±)
Light off		
0800	0	3 (FD±, N+, W+)
1000	1 (SAS-)	1 (FD±)

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TEMAZEPAM AND PERFORMANCE
FOLLOWING A SLEEP CYCLE SHIFT

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ABSTRACT

Temazepam has been recommended for diurnal and other flight crew rest periods that might be accompanied by acute insomnia. Since chronokinetic differences in benzodiazepine absorption have been reported, the effectiveness of temazepam in producing a restful, diurnal sleep without consequent effects on performance was evaluated. A repeated measures design was used to administer 3 doses of temazepam (15 mg, 20 mg, 30 mg). Sleep was delayed 12 hrs, commencing at 0700 following each dose. Subsequent performance tests were conducted after 5.5 hr of sleep from 1300 and continuing every 2 hrs until 2300 hrs. No drug effects were found on well practiced performance tests nor on mood profiles. Although all doses promoted sleep, subjects were found to be impaired in their ability to recall or recognize pictures and words following the 20 and 30 mg dose. Accordingly, the 15 mg dose is recommended as the initial dose for crew related insomnia.

KEY WORDS

Temazepam; Diurnal sleep; Performance; Memory

INTRODUCTION

Diurnal flight crew rest periods are occasionally required to ensure 24 hr support for military operations. Temazepam has been used to reduce insomnia related to atypical crew rest cycles (Nicholson, 1984; Storm, 1986) and to resynchronize travel related disruptions of sleep wake periods (Keul et al., 1988). Like many benzodiazepines, temazepam has been used as a safe and effective means to induce sleep (Irjala et al., 1989). However, like most centrally active benzodiazepine compounds, temazepam can produce anterograde amnesia (Roehrs et al., 1984). This effect seems to be less pronounced for temazepam than for more receptor specific benzodiazepines (Scharf et al., 1988).

The pharmacokinetics of the benzodiazepines are reported to be sensitive to circadian periodicity. For example, temazepam is more completely absorbed following a morning dose when compared to evening administration (Muller et al., 1987). Accordingly, this study was conducted to determine the effectiveness of temazepam in diurnal sleep maintenance and to evaluate any consequent effects on performance or memory. Since conditions in which temazepam would be prescribed for

aircrew often follow an abrupt, acute shift of the normal sleep wake cycle, the study also was conducted using sleep delayed individuals.

MATERIALS AND METHODS

The study required 4 identical sessions to complete the repeated measures ANOVA design with 4 doses (0 mg, 15 mg, 20 mg and 30 mg) of temazepam for the 10 male and 10 female subjects. Five subjects were tested at a time during each session in individual, sound attenuated workstations. During each 28 hour session, subjects arrived in the lab at 1900 hrs where performance on 2 tests, code substitution and reaction time, was practiced at intervals until 0600 the following morning. The subjects were then transported to nearby, individual sleeping quarters. At 0700, in a double blind manner, a single dose of temazepam was administered. The 20 mg dose was a soft, gelatin formulation whereas the 15 mg and 30 mg doses were hard capsule formulations. The subjects consisted of military and civilian personnel who were required to abstain from all medications as well as caffeine and tobacco dor the duration of each session. The procedures was approved by the Advisory Committee on Human Experimentation at the School of Aerospace Medicine.

The subjects were allowed to sleep until 1230 for a total of 5.5 hrs sleep. While asleep, they wore wrist mounted activity monitors to indicate the duration and extent of restlessness during sleep (Leigh et al., 1988). They were then awakened and returned to the performance lab for study. Beginning at 1300 and every 2 hrs thereafter until 2300 hrs, the subjects were given a 1 hr test sequence to measure the aftereffects of temazepam. The test sequence consisted of the 2 well practiced performance tasks and the Profile of Mood States survey (POMS). Then a set of 5 novel icons were shown followed by the presentation of 5 novel tape recorded nouns. This allowed a comparison of visually presented (icons) and auditorially presented information (nouns). Immediate recall for the items (short term memory) was assessed for the icons then for the nouns. Five minutes were given for memorization of a set of 10 numbers which was intended to prevent rehearsal of the icons and nouns. The subjects were then returned to a separate room until the next test sequence. At the final test sequence (2300 hrs), instead of a new list of the pictures or nouns to memorize, subjects were asked to 1.) recall the 25 pictures then the 25 nouns they had been given earlier and then 2.) to recognize the pictures and nouns interspersed with 25 unfamiliar pictures and nouns. The subjects were then taken home.

RESULTS

Temazepam, under the conditions of the study, was without effects on gross body movement monitored during sleep compared to placebo, suggesting a restful sleep was induced by all doses. As well, temazepam was without effects on the 2 performance tasks, code substitution and reaction time at any of the times sampled. Similarly, no drug effects on any mood dynamics as measured by the POMS inventory were found. However, short term memory for the 5 words presented during a trial was reduced by the 30 mg dose at the 6 hrs post dose and by the 20 mg and 30 mg doses at the 11 hrs post dose times as shown in Fig. 1.

As well, the same dose reduced the number of visual items retained in a delayed memory test (recall) as compared to placebo, shown in Fig. 2. Finally, the 20 and 30 mg doses were found to impair the selection of familiar nouns from the unfamiliar nouns during the recognition test, also shown in Fig. 2.

After awakening before the start of each testing session, subjects were asked to rate the quality of the sleep they experienced. On the fourth and final session, they were asked to rate the each of the 4 rest periods in the study from best to worst. These results are shown in Table 1. As shown in the table, the majority of subjects (50 %) felt that the 15 mg dose produced the most restful sleep (best)
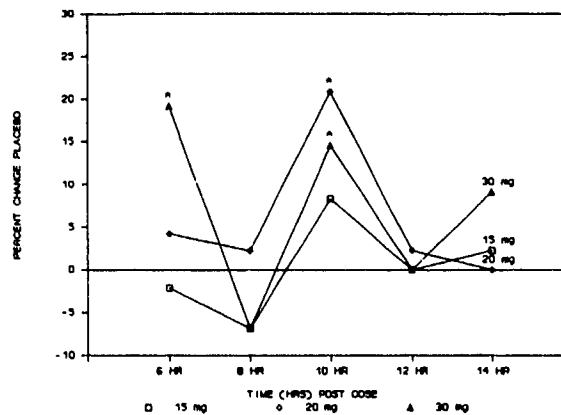
Fig. 1. Temazepam at 20 mg and 30 mg reduces short term recall of nouns (* = p <
.05) at times indicated relative to placebo.

whereas the majority (64 %) also recognized the 30 mg dose as producing the least
restful sleep (worst).
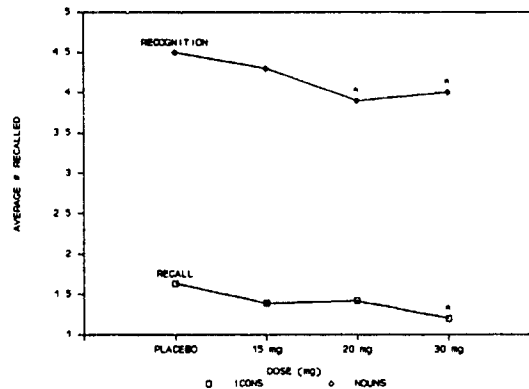
Fig. 2. Temazepam at 30 mg and 20 mg reduces icon recognition and 30 mg reduces
noun recall (* = p < .05). Five icons and five nouns were shown at each time.

The performance tests were well practiced throughout the sleep delay period and may
account for their insensitivity to temazepam during the present conditions. The
reduction in noun recall may be related to the impaired short term memory for nouns
shown in Fig. 1 suggesting that the auditory information was not encoded properly
by mneumonic processes. As well, the drug effects may have been compounded by the
effects of fatigue (since our subjects were only given a 5.5 hr crew rest after ap-
proximately a 12 hr sleep delay). We are currently evaluating this possibility.
Temazepam at the doses studied, is an effective compound for maintaining diurnal

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Table 1. Percent of subject's rating each dose by quality of sleep.

Dose	RATED BEST	RATED WORST
Placebo	21	21
15 mg	50	0
20 mg	21	14
30 mg	7	64

sleep in sleep delayed individuals. The adverse effects on memory produced by the 20 and 30 mg doses as well as the subjective ratings of quality of sleep suggest that the 15 mg dose might be the better choice for sleep induction during atypical rest periods.

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PERSISTANCE OF A CIRCADIAN RHYTHM IN AXILLARY TEMPERATURE (AT) OF ADULT EPILEPTIC PATIENTS

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ABSTRACT

Rhythmic patterns of Axillary Temperature (AT) were investigated in 8 epileptic patients: Idiopathic Generalized Epilepsy (n= 6), Complex Partial Seizure (n= 1), Undetermined Epilepsy (n= 1), (*ILAE's classification*). Circadian rhythmicity of AT persisted unchanged (7/8). These findings are clearly in opposition with results obtained by two of us in major affective disorders. In the patient with Undetermined E. , an ultradian periodicity of AT with prominent $\tau = 9.6h$ was observed; this result is discussed according to long duration of disease, multiple and various EEG epileptiform abnormalities or associated depressive factor .

KEY WORDS

Axillary Temperature , Circadian rhythmicity, EEG Paroxysmal Abnormalities, Epilepsy

INTRODUCTION

Rhythmical occurrence of seizures was recognized in Idiopathic Generalized Epilepsy (IGE) as Awakening E. (Janz, 1962) and Myoclonic jerks . Two of the authors (Monge-Strauss and Mikol, 1988) had previously reported a circadian pattern of EEG Paroxysmal Abnormalities (PA) with an awakening PA peak in IGE versus an ultradian pattern (3 peak-a-day) in cryptogenic Complex Partial Seizures (CPS) ($p < 0.002$). Two major questions can be raised : 1) is the circadian pattern of PA in IGE linked to light/darkness alternation and/or melatonin plasma changes? 2) is epileptic disease able to induce changes of endogenous rhythms ? The aim of the present study was to document rhythmic patterns of Axillary Temperature (AT) in 8 adult epileptic patients.

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PATIENTS ET METHODS

Patients

3 men, 5 women - age range from 14 to 52 y. - were investigated during a short hospitalization. The seizures were grouped according to Classification of International League Against Epilepsy. IGE : Grand Mal + EEG photosensitivity and/or myoclonic jerks (n= 6); CPS secondarily generalized (case 4); Undetermined E. (case 8). In all patients, *neurological examination* was normal. MRI displayed no space-occupying lesion, brain angioma, or significant localized atrophy, but one (case 8 : right T2-T3 atrophy). *Duration of disease* was more than 10 years in 5 subjects, *seizures frequency* more than one a week in 4. *Treatment* consisted in polytherapy (n = 5),monotherapy (n = 3): mostly phenobarbital or Na Valproate; blood levels were tested iteratively. *Routine EEG (R/EEG) recordings* were altered with Paroxysmal Abnormalities : generalized spikes or polyspikes and waves in all patients + focal spikes in 2 patients (cases 4 , 8)

Methods

*48h-EEG recordings (A/EEG)*were performed using a portable 8-channel cassette device . Data were analyzed by visual scoring (Monge-Strauss and Mikol, 1988). *Axillary Temperature* was recorded for 48h at 6mn intervals using a battery operated and portable electronic device connected to a probe (Bicakova-Rocher et al. 1989). The latter was shielded to minimize environmental changes of temperature during the recording span. Individual time series were analysed using several complementary methods : chronograms, cosinors and power spectra. *Venous blood* was sampled every 4h for 24h (7 samplings) to document rhythms of melatonin (data were not yet available at the time of this writing).

RESULTS

A/EEG data analysis showed an awakening PA peak in the 6 patients with IGE, but not in the patient with CPS : result in good agreement with our previous findings (Table 1).

Table 1.   R/EEG and A/EEG Data

Case N°	R/EEG FINDINGS			A/EEG : PA TEMPORAL OCCURRENCE			EPILEPTIC SEIZURES during A/EEG
	general PA	focal PA	photo-sensitivity	awakening	nocturnal arousals	falling asleep	
1	+		+	+		+	+
2	+		+	+	+		
3	+			+	+		
4	+	+		+			+
5	+			+	+		
6	+		+	+	+	+	
7	+			+			
8	+	+	+	+	+	+	

Patient with Undetermined E. (case 8) had generalized PA with EEG photosensitivity + focal PA. Peaks occurred at awakening, 4.30 p.m. and 1 a.m., showing mixed pattern (IGE/CPS) (fig 1)
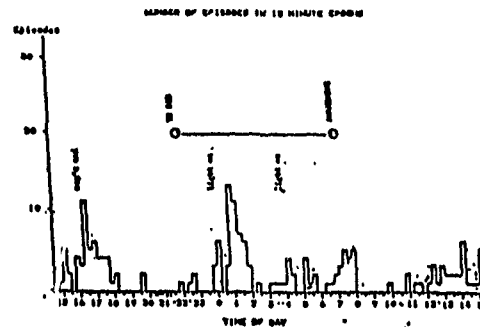
Fig. 1. case 8 : A/EEG PA temporal occurrence

Axillary Temperature characteristics : all patients with IGE as well as patient with CPS exhibited a prominent periodicity of AT with τ= 24h. (Table 2).

Table 2. Axillary Temperature characteristics resulting from time series analyses

case N°	prominent period τ (hrs) power spectrum	24 hrs mean temperature (°C) (±s.e.m.)	rhythm amplitude (°C) (95% confidence limit) cosinor	acrophase hrs
1	24	35,5 (±0,02)	0,6 (±0,009)	20.15 (±0,30)
2	24	35,9 (±0,01)	0,3 (±0,05)	20.05 (±0,30)
3	24	35,6 (±0,01)	0,2 (±0,05)	20.20 (±0,35)
4	24	35,9 (±0,01)	0,2 (±0,05)	19.30 (±0,30)
5	24	35,8 (±0,02)	0,3 (±0,06)	21.00 (±0,40)
6	24	35,7 (±0,01)	0,33 (±0,05)	17.15 (±0,45)
7	24	36,3 (±0,01)	0,26 (±0,04)	17.40 (±0,40)
8	9,6	35,3 (±0,01)	0,13 (±0,04)	23.30 (±0,35)

Patient n° 8 had a prominent τ= 9.6h. (fig 2)
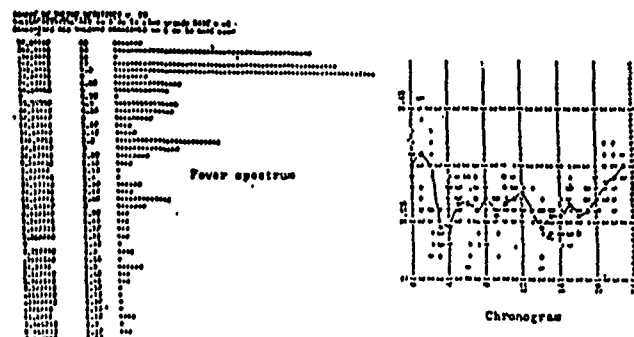
Fig. 2. case 8 : AT characteristics

CONCLUSIONS

1) In these patients with severe epilepsy, long-lasting evolution and persistent seizures
in spite of heavy treatment, circadian periodicity of AT remained unaltered except in case
n°8. These findings are clearly in opposition to results obtained by two of us in patients
with major affective disorders , where a prominent ultradian period of AT was
reported (Bicakova-Rocher et al. 1988). The same authors emphasized that shift from
circadian to ultradian periodicity is not associated with temperature alterations observed
either in various pathological states or resulting from pharmacologic or toxic effects
(Reinberg and Smolenski, 1983); herein, transient hypothermia was noted immediately
after the seizure (case 1) and the circadian periodicity persisted unchanged. *2) Patient
n°8* exhibited internal desynchronisation with a prominent τ= 9.6h, displaying
ultradian periodicity of AT. It is noteworthy that this case, the oldest of the group, was not
a typical IGE but referred to as undertermined epilepsy with focal brain atrophy, +
multiple and various EEG data .The disturbance of the thermal cycle could be hypothetized
as due to severe desorganization of rhythmic patterns underlying mechanisms of
epileptiform discharges or linked to an associated depressive factor at the time of
procedure .The discussion whether alterations of temperature rhythm result from this
form of epilepsy or the reserve remains academic with regards to our present knowledge.

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ADMINISTRATION-TIME DEPENDENT EFFECTS OF TRIAZOLAM ON COGNITIVE PERFORMANCE

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ABSTRACT

Triazolam in a dose of 0.25 mg or placebo was administered at 0830 or 2030 in a series of 13-hour duration, double-blind, crossover study sessions each separated by a minimum of 7 days. Cognitive performance was assessed hourly using the Walter Reed Performance Assessment (test) Battery. Twelve diurnally active men (23-39 yrs) were paid for their participation and rewarded with additional monetary bonuses for outstanding performances. Triazolam had a more severe detrimental effect on cognitive performance when timed at 2030 as compared to 0830 ($p < 0.05$). This was especially the case for the logical reasoning, spatial orientation and simple reaction time tasks. The deficit in performance independent of treatment time was greatest 4-7 hours after dosing.

KEY WORDS

Triazolam, chronoeffect, cognitive performance, humans, circadian rhythms

INTRODUCTION

Frequently, individuals employed in occupations requiring nighttime work rely on sleep-inducing medications to facilitate daytime rest. Too, those engaged in certain military and aerospace operations occasionally utilize such medications to help induce sleep at atypical times of the day. One agent commonly used is triazolam, a benzodiazepam derivative of relatively short half-life (1). Recent reports have implied that triazolam is associated with deficits in performance (2-4). In light of the recent concern about possible detrimental effects of triazolam on cognitive performance this investigation was devised. Of special concern was the effect of triazolam treatment time, in the morning versus the evening, on cognitive performance under conditions which preclude subsequent sleep due to unforeseen circumstances following dosing.

METHODS

Subjects: The subjects were 12 English speaking, computer literate, diurnally active (0700-2300) healthy male volunteers between 23 and 39 years of age. All were college graduates. The Horne and Ostberg Morningness-Eveningness

Questionnaire (5) revealed 5 subjects to be of neither morning nor evening type; 5 to be moderate-morning and 2 moderate-evening types. All were medically screened to rule out attention deficit, depression or anxiety and drug use and abuse. None evidenced vision or hand-eye coordination deficits. All subjects provided written consent; the study was approved by the University of Texas Health Science Center at Houston Committee for the Protection of Human Subjects. Subjects were paid an hourly rate for participation, and cash incentives were awarded for 1st, 2nd and 3rd place performance to stimulate compliance and sustain motivation throughout the investigation.

Performance Assessment Battery (PAB): Performance tests were conducted hourly using a computer generated, controlled and scored, multi-task PAB developed at the Walter Reed Army Institute of Research (6). Each subject performed his PAB inside a separate acoustically isolated room at a work station ergonomically designed to allow for individual adjustment. Performance was monitored from outside the rooms by staff members via windows. The PAB tasks were presented as follows: Wilkinson Reaction Time, Logical Reasoning ("A follows/precedes B"), Manikin (spatial orientation), Serial Add/Subtract (arithmetic/sustained attention), Choice Reaction Time and the Stanford Sleepiness Scale. Logical Reasoning and Serial Add/Subtract are indicative of the stage of central information processing concerned with integration/manipulation (linguistic/symbolic). Manikin, also reflects this central stage of information processing and is concerned with integration/manipulation of visual-spatial constructs. The Stanford Sleepiness Scale is a subjective rating of alertness/sleepiness.

Investigative Procedures: Training Phase. This phase convened two weeks prior to the first study session. Subjects completed a minimum of 12 training sessions, each of 1 hour duration, on the computerized PAB to attain an asymptotic performance level on the learning curve for the selected tasks.

Investigative Phase. This phase began immediately after training. Depending on the randomization schedule, subjects reported to the study site once per weekend, either at 1930 Friday or 0730 Sunday. Urine samples (for documenting non-use of controlled substances, e.g., alcohol and caffeine) were collected and a health questionnaire administered to document freedom from illness and medication use during the previous 7-day span. Testing began at 2000 Friday or 0800 Sunday, respectively, and continued thereafter, hourly for 13 consecutive hours. Each test session lasted 10-12 minutes and included the six computer presented tasks. Triazolam in a dose of 0.25 mg or placebo was given at 0830 or at 2030 using a double-blind, crossover study protocol. Subjects were confined to the premises where the investigation was conducted. Between test sessions, they watched video movies or television, played board games or cards, read books and magazines and/or listened to music tapes. Sleep was not allowed at any time during the 13-hour study sessions. Meals were fixed as to the time into each study session they were provided and as to the quantity and quality (protein, fat, carbohydrate) consumed. For investigations beginning at 2000, subjects reported after dinner. A snack comprised of fruit, muffin and juice was given at 0030 and 0530 (4½ and 9½ hours, respectively, into the study session). For sessions beginning at 0800, subjects reported after breakfast. Light meals were provided at 1230 (lunch) and 1730 (dinner), also 4½ and 9½ hours into the study session. Caffeine-containing products were prohibite · commencing 12 hours before and during all study sessions.

Statistical Analyses: Data were analyzed by a repeated measures analysis of variance for the main effects of treatment, time of treatment, subjects and time of testing as well as selected interactions of interest.

RESULTS

Throughput. Throughput was the primary index of performance for all tasks except the Stanford Sleepiness Scale (SSS). Throughput, the speed-accuracy

CHOICE REACTION TIME THROUGHPUT
HALCION – PLACEBO (DELTAS)

LOGICAL REASONING THROUGHPUT
HALCION – PLACEBO (DELTAS)

MANIKEN (SPATIAL ORIENTATION) THROUGHPUT
HALCION – PLACEBO (DELTAS)

STANFORD SLEEPINESS SCALE
HALCION – PLACEBO (DELTAS)
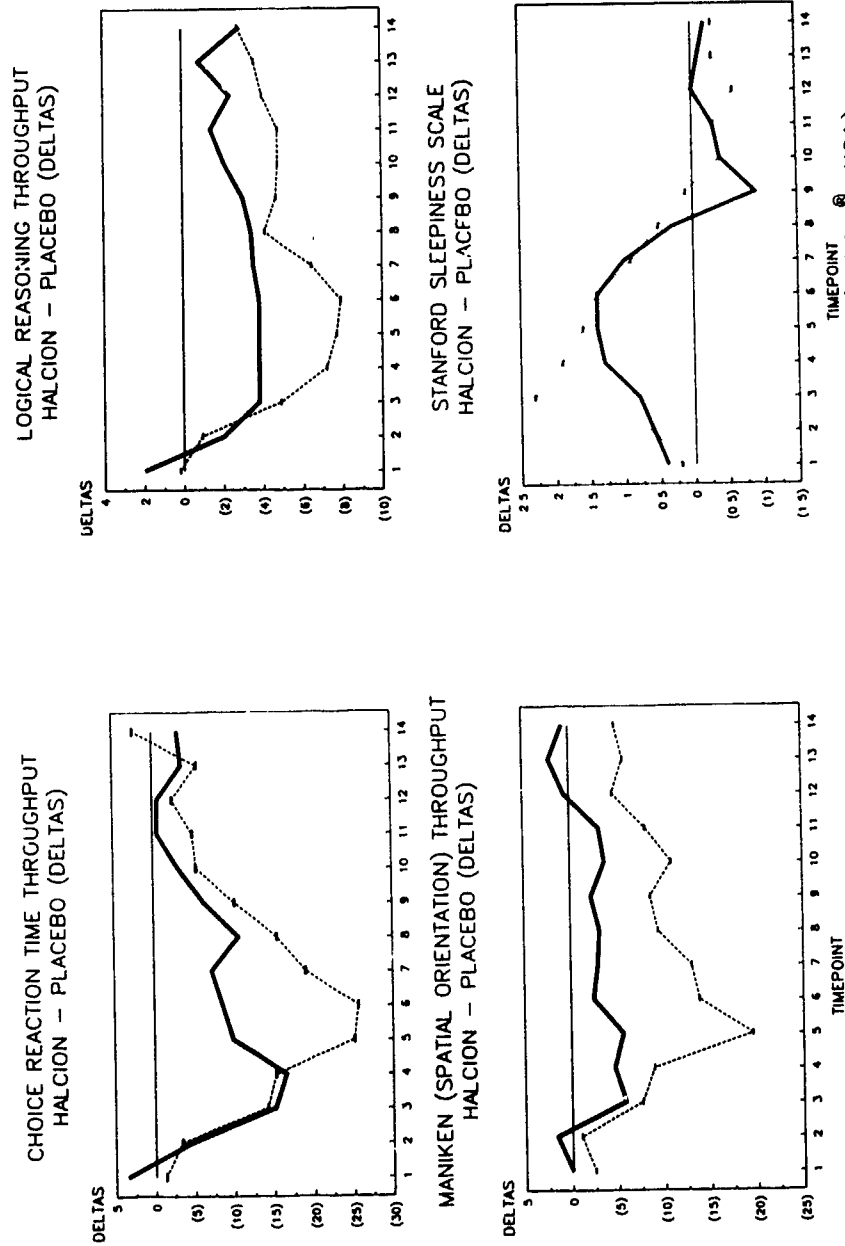
For Choice Reaction Time, Logical Reasoning and Maniken PAB tasks, triazolam (Halcion®, USA) as an evening (---) dose was associated with a greater deficit in performance than as a morning (——) dose when referenced to the corresponding-in-time placebo control values (indicated by "o" reference line). Moreover, triazolam dosed in the evening (---) produced greater loss of arousal as substantiated by the Stanford Sleepiness Scale than the morning dose (——) when referenced to the corresponding-in-time placebo control values.

product, is the number of correct responses per time span required to conduct a given trial. A high score denotes good performance, a low one poor performance. For SSS, the higher the score, the greater the subjective sleepiness.

ANOVA revealed statistical significance for the main effects of treatment (placebo vs triazolam), dosing time (0830 vs 2030), subjects and time of testing. Figure 1 illustrates that triazolam, in comparison to time-qualified placebo baseline values, had its greatest effect 4-7 hours following dosing, whether at 0830 or 2030 ($p \leq 0.05$). The dosing-time effect of triazolam was most evident in terms of the E_{max}, the maximum deficit in performance due to triazolam relative to the clock-hour, placebo-control baseline value. The E_{max} was about two-fold greater after the 2030 than the 0830 triazolam dosing for the logical reasoning, spatial orientation and simple reaction time tasks as well as the SSS ($p < 0.05$). Over the entire 13-hour study span, evening treatment induced an average 17% deficit in performance with reference to control data considering all the 5 cognitive tests together. When triazolam was dosed at 0830, the overall deficit in performance was less, about 7% with reference to the corresponding-in-time control baseline values.

DISCUSSION

The results reveal that 0.25 mg of triazolam detrimentally affects cognitive performance, whether timed in the morning or evening, in young, healthy males prevented from sleeping following dosing. The effect was more pronounced on logical reasoning, spatial orientation and simple reaction tasks. The greater E_{max} after evening relative to morning treatment may reflect the observation that benzodiazepines have a greater half-life after evening dosing (7) and a more extreme effect on arousal with reference to morning treatment. Morning-evening differences in effect also may represent the chronesthesy (circadian susceptibility) of the central nervous system to triazolam. Further study is yet required to understand the dosing-time differences of triazolam on cognitive performance. Too, the significance of these laboratory findings to field situations awaits future investigation.

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HORMONAL EFFECTS OF SLEEP DEPRIVATION IN DEPRESSED PATIENTS

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ABSTRACT

Eight hormones were measured in 3 depressed patients, on the mornings before and after a night of complete sleep deprivation. TSH increased significantly and the patients' mood was improved, but only transiently so. The concentrations of the other hormones were unchanged after sleep deprivation. The mechanism by which sleep deprivation is an antidepressant should receive more emphasis in biological psychiatry research.

KEYWORDS

Sleep deprivation; depression; TSH; other hormones; plasma

INTRODUCTION

Sleep reduction might have an important role in the occurrence of mania (Wehr et al.,1987) and sleep deprivation is an efficacious therapy for severe depression (Gerner et al.,1979). Unfortunately the beneficial effects of one night of sleep deprivation in endogenous depression are short-lasting. The mechanisms by which these beneficial effects occur are still unknown. This study evaluates changes in endocrine variables that follow a night of sleep deprivation.

METHODS

Hospitalised male depressed patients suffering from affective disorders were included in the study. They were receiving no antidepressants or neuroleptics for at least two weeks. Between 3 and 10 days after admission, they underwent one morning of blood sampling from 8h00 to 12h00 to measure cortisol, TSH, prolactin, LH, FSH, growth hormone (GH), dehydroepiandrosterone sulfate (DHEA) and ß-endorphin. Radioimmunoasssays were used. On the same evening, they were deprived from sleep during the whole night, remaining in the presence of a doctor. On the next morning,

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they were given a light breakfast without caffeine and were not allowed to go to sleep until 12h00; blood was sampled from 08h00 to 12h00 to repeat the measurement of the hormones. The patients then received clomipramine. The severity of their depression and the possible occurrence of mania were measured using two rating scales: the MADRS has a maximum score of depression of 44 and the Bech a maximum score of mania of 63. A control group of 8 normal male subjects underwent the same blood sampling procedure, but were not deprived of sleep.

RESULTS

All three patients improved significantly during the night of sleep deprivation as well as the next morning (this improvement did not persist). The MADRS scores for patients No1 to No3 were 42, 28 and 35. The next morning the scores were respectively 26, 10 and 17. No switch towards mania was observed. Patient No1 was studied a third time after remission.

After the night of sleep deprivation, there was an increase in TSH concentrations in all three patients, by a factor of respectively 1.5, 2 and 3. This was significant relative to the very high day-to-day stability observed in the normal control subjects. This increase in TSH is shown in Fig. 1. In patient No1, TSH measured on a third occasion after clinical remission was low, as low as during the day before sleep deprivation. There was a small decrease in prolactin in two patients and no changes in cortisol secretion. All other variables (LH, FSH, GH, DHEA, testosterone, ß-endorphin) were unchanged by sleep deprivation and had values within the normal range (Fig. 2

DISCUSSION

Our study was extensive as far as the number of hormones measured is concerned. In these 3 patients, as in the 8 normal controls, we observed a high level of day-to-day stability in the values of the hormones. This stability indicates a low risk of a type II error in our demonstration that only the hypothalamo-pituitary-thyroidal axis is significantly modified by sleep deprivation. Sleep deprivation might still influence other hormones than TSH, but it would need a large number of patients to describe minute changes that occur within the range of inter-individual differences.

TSH increased in all subjects and this results is consistent with recent work on the endocrine effects of sleep deprivation; most authors found increased TSH after sleep deprivation (Kasper et al.,1988; Baumgartner and Meingold, 1986). However, in rapid cyclers, sleep deprivation failed to increase nocturnal TSH (Sack et al.,1988). Kasper et al. (1988) took blood samples from 22 depressed patients at 02h00 on the nights before, during and after a single session of sleep deprivation. They found a mean increase of TSH of 50% (the concentration of prolactin decreased to one half of the baseline values measured before sleep deprivation). There was no relation between these hormonal changes and the improvement of mood. In the work from Baumgartner and Meingold (1936), there was a positive relation between the improvement in mood and the rise of TSH, but not T4, free T4, T3 or free T3. Finally, Elsenga et al. (1988) found that body temperature increased during sleep deprivation and that this increase was higher in those subjects who responded favourably to the treatment. Body temperature and TSH are two variables that change in the same direction during states of different arousal. We have evidence for this in our studies about the influence of rest and activity on circadian rhythms in normal subjects (Schulz et al., 1987).
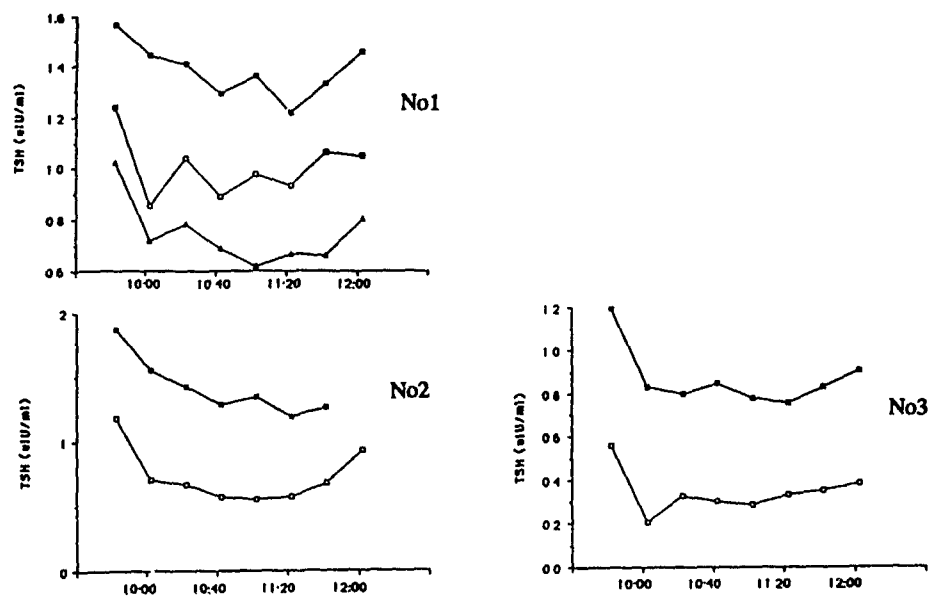
Fig. 1. TSH concentration in the morning before and after sleep deprivation
The upper curves in all three graphs indicate the concentrations of TSH during the morning after sleep
deprivation and the lower curves during the morning before sleep deprivation. One patient (No1) was
studied on a third time after remission and had lower values of TSH than during depression or after
sleep deprivation.
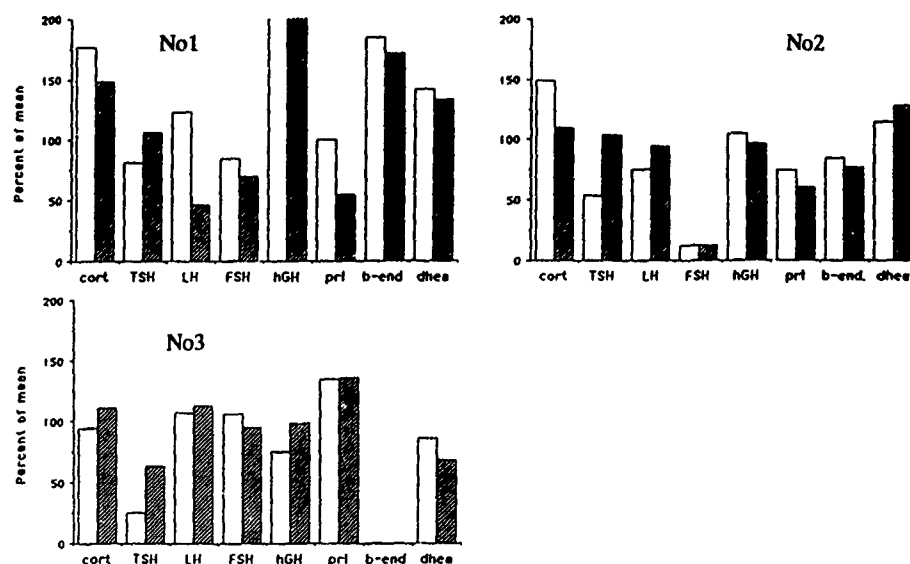
Fig. 2. Normalised mean values of hormones in three depressed patients
The values measured in these three patients are presented as percentage deviation from the means
calculated for the 8 normal control subjects. The morning before sleep deprivation is indicated by white
columns, the morning after by hatched columns. ß-endorphin was not measured in subject No3.

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The finding of increased TSH after acute mood improvement through sleep deprivation is of interest despite the fact that the concentrations of TSH before and after sleep deprivation were within the normal range in our patients, as well as in most of those studied by other authors. The hypothesis that sleep deprivation resets a higher level of activity in the hypothalamo-pituitary-thyroidal (HPT) axis is compatible with data gathered so far. However, the interpretation of the role of this change is presently difficult.

The hypothalamo-pituitary-adrenal (HPA) axis is known to be activated in many patients during severe depression, with increased concentrations of ACTH and cortisol in plasma, while TSH remains within normal values and shows a decreased amplitude of its circadian rhythm of secretion (Souêtre et al.,1988). At a purely descriptive level, it could be that sleep deprivation, by resetting the HPT and the metabolism (as reflected by changes in body temperature) at a higher level, reequilibrates the HPT activity relative to the pathologically hyperfunctioning HPA. This counterregulation being of short duration, this would explain that the clinically beneficial effects of one night of sleep deprivation do not persist.

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EFFECT OF RUBIDIUMCHLORIDE ON THE CIRCADIAN SYSTEM IN AFFECTIVE DISORDERS

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Summary

A manic-depressive 66 year old female patient was treated with rubidiumchloride in a 12-month longterm study. Besides the patient's psychic state, rectal body temperature and locomotor activity were continuously monitored in specific intervals. The time-sequence analysis showed a phase-delay relation of temperature and activity to external Zeitgebers before the treatment with rubidiumchloride. With a blood level of 0.20 - 0.40 mval/l rubidiumchloride the phase relation got normal and the psychic state stabilized at the same time.

Key Words

rubidium, lithium, circadian rhythm, affective disorders

Introduction

Rubidium (Rb^+) has an antidepressive effect. It induces improvement of mood and increases activity (1). The mode of action is unknown. Compared with lithium (Li^+), also an alcalic metal and used in the prophylaxis of affective disorders, Rb^+ shows markable differences concerning general characteristics, biochemical, physiological and pharmacological effects (2). Chronobiologically, Rb^+ induces a shortening of the circadian period in animal models (3), whereas Li^+ prolongs them (4,5). Based on the hypothesis, that the prophylactic effect of Li^+ depends on its chronobiological activity, we investigated if Lithium-Non-Responder have another basic circadian structure which would profit from a drug with phase-shortening effect. This could be Rb^+. The results of the first of several long-term studies are reported.

Material and Methods

A woman, 66 years old, suffered each year from one to two depressive episodes, type bipolar II (DSM-III-R 296,7), since she had turned 58. The episodes took generally between eight and twelve weeks. Li^+ therapy did not improve the course of her disease. We checked psychic state, rectal body temperature (by a rectal probe) and the locomotor activity at the non-dominant wrist (ZAK-Actometer) continuously before and during Rb^+ therapy over a 12 months period in defined intervals. The blood concentration of Rb^+ was determined by atomabsorptionspectrophotometry.

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There were several study intervals: before therapy with Rb$^+$ (Nov. 24 to Dec. 2, 1988),
during adjustment (Dec. 4 to Dec. 10 and Dec. 13 to Dec. 20, 1988) and during the symptom-
free interval with Rb$^+$ therapy (Sept. 28 to Oct. 8, 1989). The phase angle difference
was calculated by cross correlation between the body temperature cycle and activity
cycle.

Results

1. During the depressive episode and before Rb$^+$ adjustment we observed a phase delay
of the circadian temperature cycle as against the activity rhythm of 152 minutes (Fig. 1).
2. The test sequence analyses during adjustment to Rb$^+$ show a continuous decrease of
the phase angle difference to 99 minutes and 68 minutes (Fig. 2 and 3).
3. Continuing with Rb$^+$ therapy, a phase angle difference of 37 minutes in
the symptomfree interval was measured (Fig. 4).
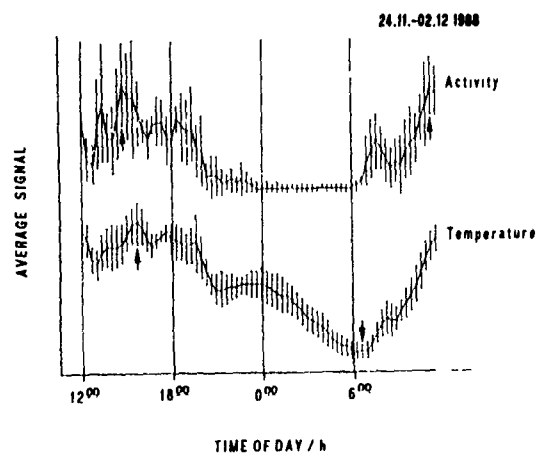
Fig.1. Circadian variation in activity and rectal temperature before Rb$^+$ in the depres-
sive episode
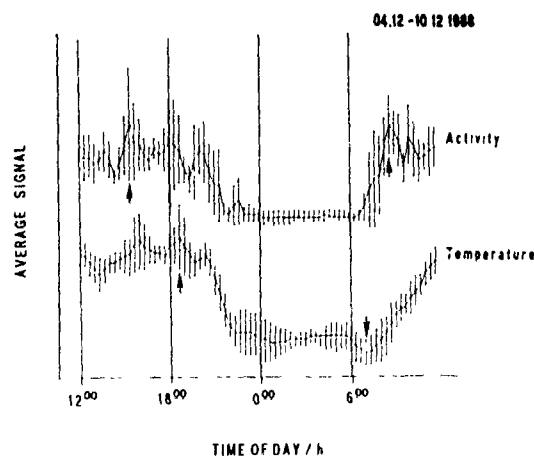
Fig. 2: Circadian variation in activity and rectal temperature during adjustment to
Rb$^+$ in the depressive episode.
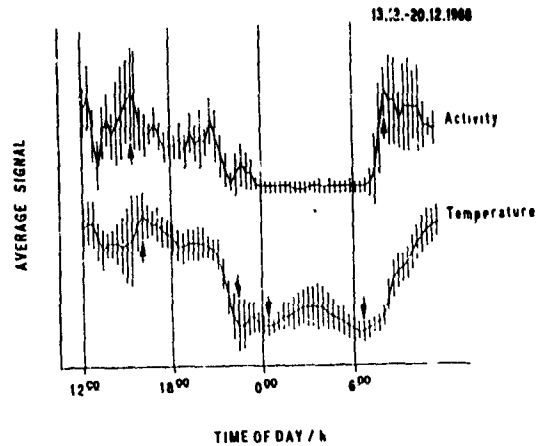
Fig.3: Circadian variation in activity and rectal temperature during adjustment to Rb⁺ in the depressive episode.
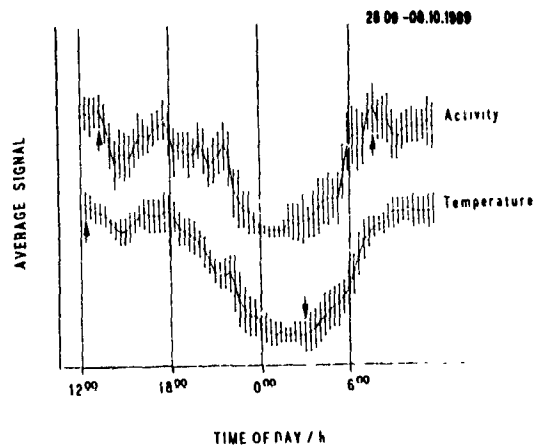
Fig.4: Circadian variation in activity and rectal temperature during Rb⁺ therapy in the symptomfree intervall.

4. At a blood concentration higher than 0.45 mmol/l, the Rb⁺ therapy was stopped because of vegetative symptoms (tendency to hyperhidrosis, nausea, slight stomach pain, freezing). There followed a continuous decline of blood concentration down to 0.05 mmol/l. In this period, the patient got depressive again. During readjustment to Rb⁺ with concentrations between 0.20 · 0.40 mmol/l, the patient got symptomfree (Fig. 5). Side effects were not repeated anymore.

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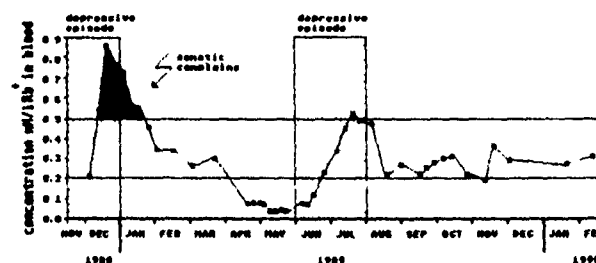
Fig. 5: Course of depression and Rb^+-concentration in whole blood.

Discussion

With Rb^+ therapy, a change of the phase angle difference occured. These observations agree with experimental studies in animals, where Rb^+ other than Li^+ shows a period shortening effect (3). The phase delay we observed could explain why Li^+ showed no effect in this patient.

Three factors could be responsible for the change in circadian parameters:

1. Rb^+ affected the circadian system (phase shortening).
2. The change in the circadian system depends only on the improvement of depression.
3. The therapeutic effect is caused by Rb^+ and its chronobiological activity.

Right now we do not know which mechanism is responsible. Further investigation and analysis in the case of this patient and of others following the same method are necessary.

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SECTION 2

ENDOCRINOLOGY AND GASTROENTEROLOGY

Chairpersons: Y. Touitou and E. Haus

CIRCADIAN VARIATION IN RESPONSE OF SALIVARY TESTOSTERONE CONCENTRATION TO TREATMENT WITH HUMAN CHORIONIC GONADOTROPHIN AT DIFFERENT CIRCADIAN STAGES IN BOYS WITH CRYPTORCHID TESTES

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ABSTRACT

Diurnally active cryptorchid boys, 11 $\pm$ 3 years of age received 1500 U U.S.P.
Human Chorionic Gonadotrophin (A.P.L., Wyeth-Ayerst) i.m. either at 0800 or at
2000, twice per week throughout a 6 week span. Salivary testosterone monitored
during this time rose in the 9 children receiving the injection at 2000
significantly more ($p < 0.001$) (+ 23.15 $\pm$ 2.39 pg/ml) than in the 10 children
receiving the treatment at 0800 (+ 2.88 $\pm$ 4.89 pg/ml), indicating a circadian
variation in Leydig cell response to injected human chorionic gonadotrophin.

KEYWORDS

Human chorionic gonadotrophin, salivary testosterone, cryptorchid boys,
circadian variation in response

INTRODUCTION

Cryptorchidism is a heterogenous entity present in about 0.8 percent of
prepubertal boys. Endocrine factors seem to play an etiologic role in more
than 50% of all cases (Job and Gendrel, 1982; Rajfer, 1986). Treatment of this
condition with human chorionic gonadotrophin (hCG) has been widely used.
However, the response to treatment with hCG varies in cases of cryptorchidism
without demonstrable anatomic obstacle between 20 and 60 percent (Rajfer,
1986; DeRosa et al., 1987; Job et al., 1987). Lagoguey et al. (1980) and
Lagoguey and Reinberg (1981) described circadian and circannual variations in
the response of the plasma testosterone concentration in healthy young adult
men to the injection of hCG. These investigators found a marked rise in plasma
testosterone within 4 hours after the injection of 2500 U of hCG at 2000. The
response was more pronounced in spring (May-June) than in fall
(October-November). No response of plasma testosterone was found to the
injection of the same amount of hCG at 0700 and only a very slight response to
the injection at 1400, which was borderline significant in spring only. We
were interested to extend these findings to the entire pituitary-testicular
axis, to boys with cryptorchid testes and to the monitoring of the testicular
response to hCG by the study of salivary testosterone. The question was raised
if the reported variability in the success of hCG in the treatment of
cryptochidism may in part be due to the difference in the response of the
patients at different circadian and circannual stages.

SUBJECTS AND METHODS

Nineteen diurnally active boys, 11 ± 3 years of age with unilateral or
bilateral cryptorchid testes were studied over a 6 week span between April 4
and May 12, 1989. Careful clinical examination by an experienced pediatric
endocrinologist showed that the boys were clinically normal in all other
aspects. Boys with evidence of anorchia, testicular ectopia and mechanical
obstacles to the descensus of the testes (e.g. hernias) and with developmental
anomalies in scrotum and penis were not admitted to the study. In questionable
cases, the testes were localized by ultrasound.

The children were followed and treated on an outpatient basis at the Section
of Endocrinology of the District Hospital of Dumbovita, Tirgoviste, Romania.
The boys were distributed randomly into two groups. Ten boys (Group 1) were
seen and treated throughout the study at midpoint 0800 and 9 boys (Group 2) at
midpoint 2000. On April 4, 7, 11, 14, 18, 21, 25, 28, and May 2, 5, 9, and 12,
1989, 1500 units U.S.P. of hCG (A.P.L., Wyeth-Ayerst) were given
intramuscularly at either 0800 or at 2000. About 2 to 3 ml of unstimulated
mixed saliva were collected immediately prior to each injection and on April
4, 11, 14, 25 and May 2 and 12, also 2 hours after the injection. Blood was
collected in EDTA immediately before and 15 minutes after the injection on
April 4, 25 and May 12.

Saliva and plasma were frozen and sent under carbon dioxide snow to the
laboratories of St. Paul-Ramsey Medical Center for the chemical
determinations. Testosterone in saliva was determined by the method of Wang
et al. (1981), interassay CV of 8.0; plasma testosterone by the method of
Kinonchi et al. (1973), interassay CV of 9.4; plasma LH by the method of Odell
et al. (1966), interassay CV of 8.1; and plasma FSH by the method of Midgley
(1967), interassay CV of 4.2. The results were evaluated by one way analysis
of variance.

RESULTS AND DISCUSSION

The salivary testosterone concentrations observed before the first injection
of hCG, were with an average of 24 ± 12 pg/ml in the group seen in the
morning, higher than in the group sampled in the evening when the average was
10 ± 3 pg/ml. This difference corresponds to the usual circadian variation.
The salivary testosterone concentrations rose in the boys treated at 2000
after the first hCG treatment in the samples collected two hours after hCG
(Fig. 1 bottom) and after the second treatment in the samples collected before
each hCG injection (Fig. 1 top), above the salivary testosterone
concentrations in the boys measured and treated during the morning hours. As
summarized in Table 1, the rise in salivary testosterone concentration over
the pretreatment values during the second to sixth week was significantly
higher in the boys sampled and injected at 2000, as compared with those
treated at 0800.

A similar difference in the testosterone response between injection at 0800
and 2000 was not obvious in plasma testosterone (Table 2). Plasma LH and FSH
were found to be markedly suppressed at the time of the sampling before the
7th and 12th hCG treatment. In the small group available and with the dose
used, the suppression of LH and FSH appeared to be similar in both groups
irrespective of the time of injection.

DISCUSSION

The observations of the time dependent changes in salivary testosterone after
injection of hCG extend the findings of Lagoguey et al. (1980) and Lagoguey
and Reinberg (1981) of a more marked response of the testes to a single
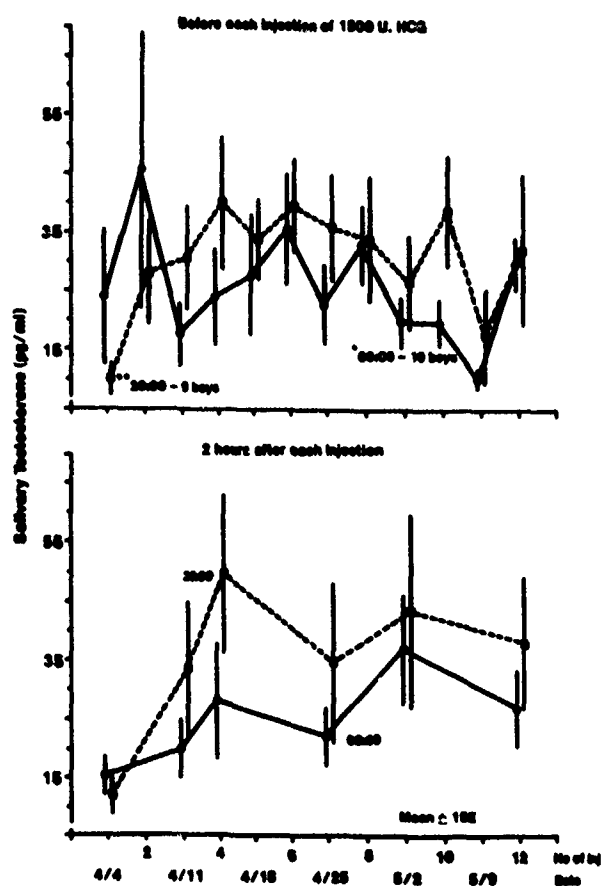
Fig. 1. Salivary testosterone concentration during a course of hCG treatment in cryptorchid boys injected either at 0800 or at 2000. Salivary testosterone in samples collected before each injection (top) and 2 hours after each injection (bottom).

Table 1. Rise in salivary testosterone concentration over the pretreatment values in cryptorchid boys receiving hCG either at 0800 or at 2000.

	Treatment Time	Salivary Testosterone		T	p
		Mean	± 1SE		
Before*	08:00	+2.56	4.99	-3.44	0.0007
	20:00	+23.15	2.39		
2 hours After*	08:00	+11.26	4.18	-2.80	0.0066
	20:00	+29.27	4.86		

* each injection of 1500 units hCG

Table 2. Plasma testosterone, LH and FSH concentrations in cryptorchid boys receiving 1500 U U.S.P. of hCG either at 0800 or at 2000.

Parameter	Treatment Time	No of Subj	Sampling before hCG injection					
			1st		7th		12th	
			Mean	± 1SE	Mean	± 1SE	Mean	± 1SE
Testosterone	08:00	10	0.382	0.119	2.528	0.687	3.904	0.867
(ng/ml)	20:00	9	0.701	0.245	3.306	0.588	3.590	1.246
LF	08:00	10	0.372	0.161	0.026	0.011	0.044	0.024
(mIU/ml)	20:00	9	0.491	0.239	0.010	0.001	0.010	0.001
FSH	08:00	10	2.640	0.791	0.990	0.090	0.928	0.038
(mIU/ml)	20:00	9	3.289	0.782	0.811	0.089	0.740	0.160

injection of hCG in the evening in clinically healthy young adult subjects, to a prolonged course of treatment with hCG in boys with cryptorchid testes. Our findings also indicate the feasibility to use salivary testosterone as a gauge to measure the testicular response. In plasma collected only at two time points during the treatment span, such a time dependent difference in response was not obvious in total testosterone. The suppression of LH and FSH was marked in both groups. A slightly more pronounced suppression after the injection at 2000 was statistically not significant. It, thus, appears that in the cryptorchid boys studied, the circadian variation in testicular response to injected hCG in the dose and with the study design used, is primarily observed in the free testosterone fraction, which the salivary testosterone concentrations are thought to represent.

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THE EFFECT OF CORTICOSTERONE
ON THE PIGEON CROPSAC RESPONSE
TO PROLACTIN

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ABSTRACT

The circadian rhythm of cropsac response to prolactin (0.1 mg ovi-
ne prolactin/day, 31 I.U. per mg) was studied in young pigeons
Columba livia maintained on constant light. One control group of
pigeons received prolactin only for 4 days at either 0600, 1200,
1800, or 2400. Uninjected controls were also included. In other
birds, corticosterone (0.3 mg/day) or saline injections were
given at either 0600, 1200, 1800, or 2400 in an effort to reset or
phase the rhythm. After 2 days of corticosterone or saline injec-
tions, prolactin injections were made at either 0600, 1200, 1800
or 2400. Thus, 0, 6, 12, and 18-hr temporal relations were estab-
lished. The pigeons were killed 24 hours after their last prolac-
tin injections and the cropsacs were removed and weighed. The
rhythm of cropsac response largely remained synchronized to the
clock time of the day (peak response at 2400). However, pigeons
which received prolactin injections in various temporal relations
with corticosterone also showed a possible phasing effect of cor-
ticosterone as seen in the 18-hr temporal relation giving the
greatest response.

KEY WORDS

Circadian rhythm; Columba livia; cropsac response; prolactin;
corticosterone; temporal relation.

INTRODUCTION

Marked circadian rhythms in pigeon cropsac responses to prolactin
have been described (Burns, 1968; Meier and Burns, 1968; Burns and
Meier, 1971; Meier, et al., 1971a; Burns and Larson, 1990). In
general, these studies show that the response to a series of daily
prolactin injections is low when injections are given at the onset
of the photoperiod, high in the early afternoon to early night,
and moderately high during the late night.

Corticosterone injections have been reported to phase the circ-
adian rhythm of cropsac response to prolactin in pigeons that were
kept on constant light. Thus, the greatest cropsac response occur-

red when prolactin was given daily 18 hours after injections of
corticosterone, no matter whether the corticosterone was given at
0600 or 1800 (Meier, et al., 1971a). However, in two other simi-
lar studies groups of pigeons receiving corticosterone at 0600 or
1800 responded only partially as expected to prolactin given in
various temporal relations (Meier, et al., 1971b, 1971d). Because
of the importance of the concept of a temporal synergism of corti-
costerone and prolactin in explaining seasonality in vertebrates
(Meier, et al., 1971c, 1978, 1981; Burns and Meier, 1988) we
sought more information.

MATERIALS AND METHODS

In October, 171 silver king pigeons Columba livia (8 - 10 weeks
old) of mixed sexes arrived at Bethany College from South Caro-
lina. The pigeons were kept on constant light (150 - 200 lux at
cage level) and were given water and grain continuously. After two
weeks the injections were begun. The daily injections were 0.85%
saline, 0.3 mg corticosterone, or 0.1 mg ovine prolactin (31 I.U.
per mg). Corticosterone or saline was given daily for 6 days and
prolactin was given daily for 4 days beginning on the 3rd day. The
number of birds were: 4 uninjected; 24 prolactin only (6 at 0600,
6 at 1200, 6 at 1800, and 6 at 2400); 48 saline and prolactin (12
with 0-hr, 12 with 6-hr, 12 with 12-hr, and 12 with 18-hr rela-
tions between saline and prolactin injections - each group of 12
was subdivided into 4 subgroups of 3 birds which received saline
at 0600, 1200, 1800, or 2400); 95 corticosterone and prolactin (24
with 0-hr, 23 with 6-hr, 24 with 12-hr, and 24 with 18-hr rela-
tions between corticosterone and prolactin injections - each group
of 24 was subdivided into 4 subgroups of 6 birds which received
corticosterone at 0600, 1200, 1800, or 2400). The pigeons were
killed 24 hours after their last prolactin injection and the crop-
sacs were removed and weighed.

RESULTS

See Table 1. The prolactin only group showed a circadian rhythm
of response that was statistically significant by a one way ANOVA
(P $<$.005) and by a fit to a cosine curve (P$<$.033). The saline and
prolactin groups, analyzed according to the time of prolactin in-
jections, showed a rhythm of response that was statistically sig-
nificant by a one way ANOVA (p$<$.0001). In both cases, the great-
est response to prolactin occurred at 2400. In general, birds
that received saline and prolactin showed a greater response than
birds that received corticosterone and prolactin (P$<$.025). The
corticosterone and prolactin groups, analyzed according to the
time of the prolactin injections, showed a rhythm of response that
was statistically significant by a two way ANOVA (p$<$.0001). There
was no significant variation according to the time of the cortico-
sterone injections. The saline and prolactin groups, analyzed ac-
cording to the temporal relation between injections, diagonals in
the table, gave responses that did not vary significantly by a two
way ANOVA (p$>$.25). The corticosterone and prolactin groups, ana-
lyzed according to the temporal relation between injections, dia-
gonals in table, gave responses that did vary significantly by a
two way ANOVA (p$<$.005). The largest response was found in the
18-hr temporal relation.

DISCUSSION

The cropsac responses of the pigeons largely remained synchronized

Table 1. Pigeon cropasc responses (g) to various temporal relations of saline or cortico-sterone and prolactin injections.

	TIME OF PROLACTIN INJECTION			
	0600	1200	1800	2400
	1.83 ± .17	1.88 ± .14	1.79 ± .18	3.27 ± .31
All Values: 2.19 ± .16				

Cortico-steroid

	0600	1200	1800	2400
0600	1.52 ± .06A	1.50 ± .14B	1.47 ± .14C	3.53 ± .50D
1200	2.06 ± .27D	1.15 ± .08A	1.72 ± .11B	2.73 ± .19C
1800	2.19 ± .28C	1.98 ± .31D	1.58 ± .10A	2.27 ± .22B
2400	1.80 ± .12B	1.93 ± .27C	1.73 ± .13D	2.59 ± .28A
Total	1.90 ± .11	1.64 ± .12	1.62 ± .06	2.78 ± .18

Temporal relations of corticosterone & prolactin:
      A (0 -hr) = 1.71 ± .13; B (6 -hr) = 1.82 ± .10;
      C (12-hr) = 2.08 ± .14; D (18-hr) = 2.32 ± .21.

All Values: 1.98 ± .08

Saline

	0600	1200	1800	2400
0600	2.83 ± .24A	1.08 ± .09B	2.07 ± .35C	3.25 ± .36D
1200	2.70 ± .15D	2.12 ± .19A	1.83 ± .62B	3.33 ± .38C
1800	2.95 ± .22C	1.93 ± .38D	1.72 ± .04A	3.13 ± .07B
2400	1.70 ± .25B	1.67 ± .20C	1.83 ± .23D	2.65 ± .10A
Total:	2.55 ± .18	1.70 ± .16	1.89 ± .18	3.09 ± .14

Temporal relations of saline & prolactin:
      A (0 -hr) = 2.33 ± .15; B (6 -hr) = 1.94 ± .27;
      C (12-hr) = 2.50 ± .24; D (18-hr) = 2.44 ± .21.

All Values: 2.30 ± .11

Uninjected: 1.39 ± .19

to the clock time of the day (peak at 2400), as seen in the pro-
lactin only group and in the other two groups as well. Also, the
temporal relation of corticosterone and prolactin (0-hr, 6-hr,
12-hr, or 18-hr) made a difference in the response obtained, with
the 18-hr temporal relation giving the largest response. In early
studies, the injection of corticosterone at 0600 followed by pro-
lactin at 2400 (an 18-hr relation) may have given an unusually
large response due to the peaking of both rhythms phased by corti-
costerone and underlying rhythms synchronized to the clock time of
the day. More evidence is needed to conclude that corticosterone
has its effects by resetting or phasing a circadian rhythm of crop-
sac sensitivity to prolactin, but this study is compatible with
that conclusion.

ACKNOWLEDGMENT

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EFFECTS OF A SEROTONINERGIC AGENT, d-FENFLURAMINE, ON THE CIRCADIAN RHYTHM OF PROLACTIN, ACTH AND CORTISOL

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ABSTRACT

d-Fenfluramine (dF) administration at therapeutic doses did not modify either the circadian chrono-organization of prolactin, ACTH and cortisol secretion, or the adrenocortical responsiveness to exogenous corticotropin. However, single doses of d-F may induce small and transient increases of plasma levels of PRL, ACTH and cortisol. The acute effects of d-F seemed to be higher in control subjects than in obese patients.

KEY WORDS

d-Fenfluramine, prolactin, ACTH, cortisol, circadian rhythm, Synacthen test.

INTRODUCTION

The anorectic activity of d-fenfluramine (d-F) arises from its capacity to stimu= late the release of serotonin and to inhibit the serotonin reuptake in the CNS (Fuxe et al,1975; Garattini et al, 1975).

Due to its effects on the central serotoninergic tonus, d-F given at pharmacologi= cal doses reportedly induces significant changes of different pituitary secretions both in experimental animals and in humans (Lewis and Sherman, 1984, 1985; Van de Kar et al, 1985).

The aim of our study was to evaluate the effects of acute or sustained administra= tion of therapeutic doses of d-F on the circadian chrono-organization of prolactin and ACTH-cortisol secretion.

SUBJECTS AND METHODS

The study involved 8 clinically healthy women aged 24-30 y. ($\bar{x}$=26.8+.2 SEM) and 10 obese women aged 17-33 y. ($\bar{x}$=23.8+1.7), with BMI 26.3-47.6 Kg/m^2 ($\bar{x}$=33.7+2) and per cent overweight from the I.B.W. ranging from 20.5 to 119.6% ($\bar{x}$=53.4+9.7). Menstrual function was regular in controls as well as in obese patients.

The group of obese subjects was homogenous for fat topography, being "android" (with predominant fat accumulation in the upper part of the body) or "intermedia= te" (with fat accumulation both in upper and lower part of the body).

Both the obeses and the controls were studied as in-patients, with free choice of diet and with the same synchronization to the hospital life schedule (meals at 08^{00}, noon and 18^{00}; sleep in darkness 21^{00} to 07^{00}, approximately). No drugs were given for 10 days at least before the study.

The effects of acute administration of a single dose of d-F were studied in healthy

controls as well as in obese patients, by the evaluation of :

1. the circadian rhythm of plasma PRL, ACTH and cortisol under basal conditions (18th day of the menstrual cycle) and after d-F 30 mg given at 17^{00}or at 05^{00}(re= spectively 19th and 24th day of m.c.);

2. the cortisol response to pulse i.v. injection od 2,500 ng of β 1-24 synthetic corticotropin (Synacthen) given respectively at $08^{30}$and $20^{30}$of the 15th and 16th day of m.c. in basal conditions, and at 20^{30} and $08^{30}$of the 5th and 10th day of the following menstrual cycle (after administration of d-F 30 mg respectively at $17^{00}$and 05^{00}).

Moreover, the same evaluations were repeated in obese patients in corresponding days of the m.c., after 30 days of d-F administration 15 mg twice a day (at 08^{00} and 20^{00}), in order to evaluate the neuroendocrine effects of d-F given according to a therapeutic schedule.

Blood samples for rhythmometric study were drawn every 4 hours during the day and every 2 hours during the night, through an indwelling catheter inserted in an ante cubital vein. Plasma was stored at -20°C until assayed.

Plasma hormones were measured by RIA methods, using commercial kits.

The statistical analysis of data was performed by conventional methods (mean,SEM, paired Student's t test) and by the single and population mean cosinor method (Halberg 1969).

RESULTS

a) Rhythmometric study

The Table summarizes the data recorded in healthy controls and in obese patients before and after d-F.

The administration to healthy controls of a single dose of 30 mg of d-F at 17^{00}in= duced a significant increase of plasma PRL levels during the following night and again 19 and 23 hours after the administration. When the same amount of d-F was given in the morning, plasma PRL levels tended to be higher than in basal condi= tions, but differences did not reach statistical significance. The circadian pro= file of plasma PRL exhibited in obese patients the same trend as in healthy con= trols, both in basal conditions and after the single dose of d-F, but without any statistical significance of the differences between the basal and the post-dF obser vations. After 30 days of treatment, the PRL circadian profile was very similar to the one of basal conditions, even though with a tendency to higher levels both during day- and night-time. The difference reached statistical significance only for samples collected at $08^{00}$and at 20^{00}. The population mean cosinor method vali= dated the statistical significance of PRL circadian rhythm in healthy controls both in basal conditions and after d-F administration, without significant changes of mesor, amplitude and acrophase. In obese patients the circadian rhythm of pl. PRL reached statistical significance under basal conditions but not after acute or sustained d-F administration.

Both in healthy c   rols and in obese subjects a significant increase of the noctur= nal levels of pl. ACTH occurred after 30 mg of d-F given at 17^{00}. The same dose of d-F given at $05^{00}$was followed by a significant increase of ACTH nocturnal levels in healthy controls but not in obese patients. Besides, the 30 day treatment by d-F did not induce any significant changes of the circadian profile of pl.ACTH. In healthy controls, plasma cortisol levels recorded throughout the 24 h. cycle after d-F 30 mg at 17^{00}were nearly superimposable to that found in basal conditions, except for a slight increase during night-time. When d-F was given in the morning, a significant increase of plasma cortisol levels occurred in the following evening. In obese patients plasma cortisol levels after a single dose of d-F tended to be higher than in basal conditions, but the differences reached statistical signifi=

cance only for values recorded in the night-hours following d-F administration in the afternoon. No significant changes of the circadian profile of pl.cortisol we= re apparent after 30-days' treatment by d-F.

At the population mean cosinor analysis the ACTH circadian rhythm in healthy con= trols reached statistical significance only after acute d-F administration and in obese patients only after the 30-day treatment. On the contrary, the circadian rhythm of pl.cortisol was detectable both in basal conditions and after d-F admi= nistration in both groups of subjects. Healthy controls showed a significant increa se of the mesor of cortisol circadian rhythm after d-F at $05^{00}$and of the amplitu= de of the same rhythm after d-F at 17^{00}. Obese patients exhibited a significant increase of cortisol circadian mesor after the administration of d-F at 17^{00}.

b) Synacthen test

The adrenocortical responsiveness to pulse stimulation by synthetic corticotropin was not significantly modified by d-F, and the chrono-sensitivity of the adrenal cortex to exogenous corticotropin was maintained. In fact both in basal conditions and after acute or sustained d-F administration the amplitude of cortisol response to Synacthen was significantly higher in the evening than in the morning.

CONCLUSIONS

The acute administration of a single dose of d-F was followed by small increases of plasma levels of PRL, ACTH and cortisol, in agreement with other data in the literature (Lewis and Sherman, 1984, 1985).

The effects on plasma PRL, ACTH and cortisol levels of changes of the central se= rotoninergic pattern induced by d-F were generally more evident when the single dose of the drug was given in the evening, before the "critical" phase of the circadian activation of the hypophyseal-adrenal axis. These results agree with previous data in the literature, concerning the role played by serotoninergic pathways on the circadian chrono-organization of the pituitary-adrenal system (Hiroo Ymura et al, 1973; Szafarczyk et al, 1980).

When compared with healthy controls, obese patients seemed to exhibit a reduced sensitivity to serotoninergic changes induced by d-F, and this finding may further support the hypothesis of impaired central serotoninergic tonus in obesity (Wurtman,1986). However, d-F given at therapeutic doses did not induce any signi= ficant alterations of the circadian secretory program of PRL, ACTH and cortisol. No important side-effects were observed during the 30-day d-F treatment of obese patients, who obtained a mean decrease of body weight of about 5%, without any caloric restriction. Moreover, changes of hormonal parameters observed after sustained administration of d-F were generally smaller than after a single dose of the drug, suggesting that chronic treatments may lead to tolerance phenomena, possibly reducing the effects of the drug on the serotoninergic system.

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Circadian hormonal values recorded before and after d-F administration ($\bar{x}$ + SEM)

	20	22	24	2	4	8	12	16	20
pl. PRL ng/.									
CONTROLS									
Baseline	8.3+1.2	8.4+1.3	10.0+1.4	23.9+2.6	21.9+1.7	16.6+3.1	6.1+2.1	8.0+1.2	8.2+1.0
d-F h 17[00]	9.7+1.4	11.3+2.9	16.5+3.7 *	19.3+2.3	21.6+3.4	15.7+1.1	7.5+1.4	11.9+1.5 ***	11.3+1.6 *
d-F h 05[00]						26.8+6.9	7.2+1.2	11.6+2.9	(10.8+1.8)
	10.8+1.8	13.6+3.2	9.9+1.3	23.5+3.2	21.6+2.3	20.8+3.5			
OBESE WOMEN									
Baseline	16.4+4.0	16.6+4.3	18.1+3.7	28.2+4.6	23.9+4.4	16.5+4.7	14.6+4.5	18.4+4.1	16.2+3.7
d-F h 17[00]	18.8+4.3	17.3+4.5	22.2+4.8	25.0+5.0	26.4+6.2	19.3+4.1 *	15.4+3.7	21.5+4.0	21.3+3.6
d-F h 05[00]						21.5+6.0	16.4+4.1	.7.8+5.1	(20.9+6.3)
	20.9+6.3	21.8+8.5	16.5+4.6	25.4+5.5	22.4+3.8	20.2+6.2			
d-Fx30 d.	22.3+5.6 *	17.6+3.9	21.6+3.2	26.5+5.5	23.5+5.3	21.5+4.7 *	17.6+3.9	20.4+4.0	20.8+4.7
pl. ACTH pg/ml									
CONTROLS									
Baseline	27.8+3.2	24.8+3.8	24.0+3.0	21.7+2.4	24.2+3.9	34.6+6.6	31.1+5.9	29.4+6.3	26.9+3.5
d-F h 17[00]	34.1+4.8 *	28.9+3.1	28.7+4.3	23.6+2.6	40.3+7.8 *	47.3+6.1	39.0+4.4	37.7+5.9	33.2+4.2
d-F h 05[00]						60.1+14.4	38.6+5.5	33.7+4.3	(30.8+4.2)
	30.8+4.2	26.2+3.4	25.2+4.0	23.1+2.4	33.7+6.3 **	42.1+5.9			
OBESE WOMEN									
Baseline	48.2+7.8	39.9+6.1	33.7+6.3	30.6+6.9	44.3+8.6	55.6+10.7	57.1+8.7	54.1+8.3	47.7+7.2
d-F h 17[00]	44.5+7.9	50.4+7.9	43.7+7.5	47.2+9.1 **	52.6+13.3	53.5+10.1	60.8+7.7	48.8+7.8	43.7+6.7
d-F h 05[00]						54.1+9.3	53.1+9.1	44.0+7.9	(39.7+7.8)
	39.7+7.8	42.9+7.7	35.9+7.8	44.1+10.9	43.3+11.6	47.7+9.2			
d-Fx30 d.	45.4+7.2	40.8+7.6	39.5+6.4	41.2+7.1	51.1+9.0	55.2+9.2	56.2+9.0	51.7+9.8	42.3+7.7
pl. CORTISOL mcg/dl									
CONTROLS									
Baseline	5.7+ .9	3.9+ .5	2.8+ .4	3.8+ .7	7.8+2.7	20.4+2.1	11.2+1.4	8.5+ .9	5.5+ .8
d-F h 17[00]	7.6+ .6	5.7+ .6	5.2+ .8 *	4.2+ .7	6.9+1.5	19.9+2.1	11.6+ .9	8.2+ .9	7.7+ .3
d-F h 05[00]						23.3+ .7	14.4+1.4	9.9+ .8	(10.1+1.8)
	10.1+1.8	6.8+ .8 *	4.5+ .5 **	3.5+ .4	7.6+1.4	18.7+ .9			
OBESE WOMEN									
Baseline	6.5+2.1	3.1+ .3	2.2+ .2	3.2+ .9	6.3+1.3	14.6+1.0	10.1+1.2	9.2+1.0	6.3+1.2
d-F h 17[00]	6.9+1.0	4.8+ .7 **	4.7+ .8 **	6.0+1.6	5.5+1.1	18.7+2.5	9.8+1.3	10.8+1.5	7.5+1.1
d-F h 05[00]						15.5+1.6	11.9+1.3	10.0+1.4	(9.8+3.0)
	9.9+3.0	6.5+1.8	4.0+1.0	3.9+ .7	5.7+1.2	15.7+3.1			
d-Fx30 d.	6.3+1.4	4.2+1.0	4.1+1.8	5.3+2.1	5.5+ .8	17.3+2.5	8.6+1.2	8.5+ .7	5.7+ .6

Paired Student's "t" test (vs baseline) : * p (.05   ** p (.01   *** p (.001

CHANGES OF THE CIRCADIAN PATTERN OF PLASMA MELATONIN RELATED TO AGING

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KEY WORDS
Aging, melatonin, ACTH, cortisol, circadian rhythm

Neuroendocrine changes occurring with aging may be ascribed to different factors. In fact, they may depend on the neuronal regressive changes particularly evident in some regions of the C.N.S. (such as hippocampus and limbic system) (Sapolsky et al, 1986) ; or they may stem from the imbalance among different neurotransmit= ters in the C.N.S., due to the selective impairment of noradrenergic pathways in aging (Hornykiewicz,1983).

A clinically reliable tool to investigate the central noradrenergic tonus may be the study of melatonin circadian pattern, since the critical melatonin secretion during the night is mediated by noradrenaline through the β_1-receptors (Reiter, 1976) and may be selectively inhibited by β-adrenergic blocking drugs (Vaughan et al, 1976; Cowen et al, 1983).

Our experience in this field concerns a group of 14 clinically healthy women aged 66-90 y., in whom we simultaneously studied the circadian rhythm of plasma melato= nin, ACTH and cortisol, in order to investigate the relationships between melato= nin secretion and pituitary-adrenal axis in physiological aging.

The study was carried out in different months and not in a defined season. Plasma melatonin was measured by RIA (kit Eurodiagnostic B.V., Apeldoorn (NL)) after extraction by diethylether; plasma ACTH and cortisol were measured by direct RIA (commercial kits).

The details of the experimental procedure and the analysis of results are being published elsewhere. In the present paper we wish to focus on the major findings of the study.

A statistically significant circadian rhythm (population mean cosinor analysis) was detectable in old subjects for plasma melatonin, ACTH and cortisol. When compared to young controls (13 clinically healthy women aged 20-30 y.), old subjects exhibited higher plasma levels of ACTH and, to a lower extent, also of cortisol, both during day- and night-time. On the contrary, the circadian pro= file of plasma melatonin was clearly flattened in old subjects. In particular, old subjects showed a significant reduction of the melatonin nocturnal peak and a wide scattering of the circadian crest-time of plasma melatonin. Both parametric and non parametric regression analysis showed that the decrease of

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melatonin nocturnal peak in old subjects was significantly correlated with the
circadian mesor of plasma cortisol.

The impairment of melatonin nocturnal secretion in aging, by the way already re=
ported in the literature (Touitou et al, 1981; Iguchi et al, 1982; Sack et al,
1986), may be considered an expression of the reduction of the central noradrener=
gic tonus or of a reduced sensitivity of the pineal β-adrenergic receptors to NE
(Greenberg and Weiss, 1978).

Furthermore, the impairment of melatonin secretory pool may be contributory to
the increased ACTH and cortisol secretion in aged subjects, due to the possible
role played by the pineal gland in inhibiting the pituitary-adrenal axis (Wetter=
berg, 1983) and in modulating the adrenocortical responsiveness to corticotropin
(Sanchez de la Pena et al, 1983).

The study of melatonin circadian pattern might be useful for the clinical evalua=
tion of the central noradrenergic activity in aging.

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RELATIONSHIPS BETWEEN ENDOCRINOLOGICAL PATTERNS AND NUTRITIONAL RHYTHMS DURING THE MENSTRUAL CYCLE IN DISTURBED AND NONDISTURBED EATERS

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ABSTRACT

Daily calorie intake, ratings on hunger and mood as well as blood levels of estradiol (E_2) and progesterone (P) obtained during one menstrual cycle were compared in 40 women classified as disturbed (D) and nondisturbed (ND) eaters by questionnaires. Hunger ratings in both groups and correlations between aggressive mood and consumption of fat in D were highest at the time of P max and corpus luteum deficiencies more frequent in D than in ND. Disturbance in LH release in D subjects is discussed as an underlying mechanism.

KEYWORDS

Eating disorders, menstrual cycle, estradiol, progesterone, calorie intake

INTRODUCTION

Many studies have shown that decreased or irregular intake of food as in anorexia nervosa or bulimia may lead to irregularities of the menstrual cycle, frequently even to amenorrhea and infertility (Pirke et al., 1987, Fichter & Pirke, 1989). Experimental starvation in healthy women (Schweiger et al., 1989) revealed that reduced intake of calories leads to partial or total suppression of LH and FSH and to a reduced release of estradiol (E_2) and progesteron (P). On the other hand, reproductive hormones may be involved in the development of hunger and satiety, as indicated by observations of changes in appetite and eating patterns in pregnancy and in women on oral contraceptives (Hill et al., 1989). Furthermore, abnormal eating patterns as well as dysphoric mood have been reported to occur premenstrually in concomitance with the decrease of E_2 and P (Clare, 1985).
Therefore, mutual relationships between disturbed eating behavior, actual phase related food intake, emotional states, and endocrinological characteristics of the menstrual cycle (mc) were investigated in the present study by answering the following questions: 1. Will appetite, calorie intake, and dysphoric

mood be increased during the luteal phase of the mc and will these intracyclic differences be more
pronounced in subjects with abnormal eating patterns? 2. Are abnormalities in levels and ratios of
estradiol (E_2) and progesterone (P) more pronounced in subjects with disturbed eating patterns?

METHOD

40 healthy females aged 20-37 years were selected according to above and below mean values on a
questionnaire of disturbed patterns of eating habits (Pudel, 1985) and were requested to fill in a que-
stionnaire on restraint or disinhibited eating (Stunkard & Messick, 1985) and a diary on food intake
and to measure basal body temperature on each day of one mc. Blood samples for determination of E_2
and P by RIA as well as self ratings on emotional states (v. Zerssen, 1970) and hunger were obtained
on days 5,9,12,14,16,18,21,24,26 and 28 of the mc. Days of the cycle were synchronized according to the
day of ovulation which was determined by basal body temperature and levels of E_2 and P. Six phases of
the cycle were defined (early and late follicular, ovulatory, early luteal, day of P_{max} = mid luteal, and 3
days prior to menstruation) and compared by analysis of variance with respect to calorie intake, hun-
ger, and 6 scales of dysphoric mood.

For each phase correlations were computed between mood and total calorie intake. Furthermore,
average values of E_2 for the follicular phase, of P and E_2 for the luteal phase and their ratio as well as
of P_{max} were computed individually for comparison between groups of eating disturbance.

RESULTS

1. Eating and mood: As shown in Fig. 1., both disturbed and nondisturbed eaters showed a significant
increase in their scores on the 5 point hunger rating scale during the mid luteal phase ($p < .001$ for the
total group) but did not differ with respect to intracyclic variations of hunger.
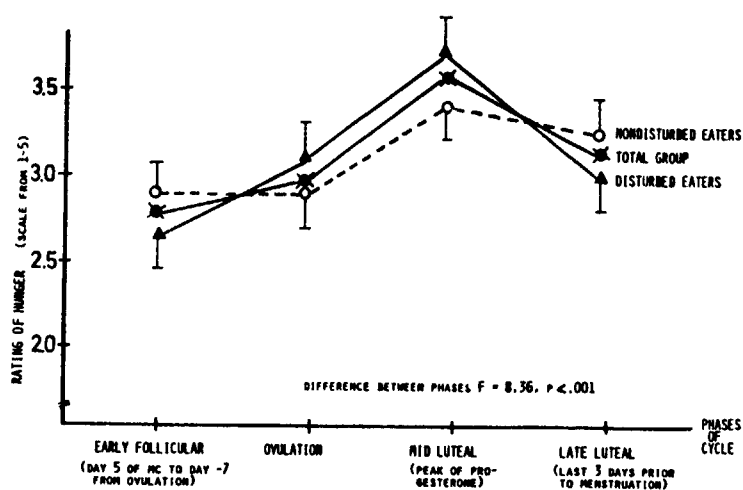
Fig. 1: Means and SEM of average daily ratings of hunger during 4 phases of the cycle

Although total calorie intake and respective consumptions of fat, carbohydrates, and proteins showed
slightly different patterns across the cycle in disturbed and nondisturbed eaters, these differences were
not statistically significant. However, polynomials computed for each group across a period of -9 to +9
days from ovulation (for which observations of calorie intake were available from 36 ovulating sub–

jects) revealed that women with disturbed eating patterns exhibit their maximum of calorie intake in the early luteal phase while nondisturbed subjects show a peak on the days of their highest P values and a smaller one in the early follicular phase (Fig. 2).
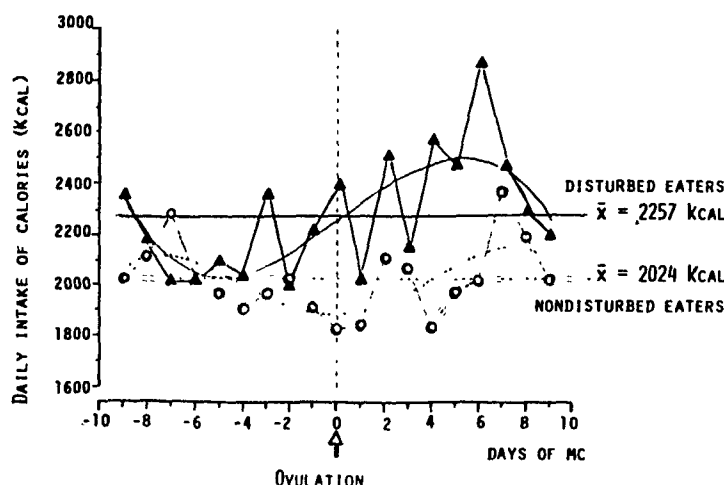
Fig. 2: Polynomials computed for total calorie intake in the disturbed (▲—▲) and nondisturbed eaters (o—o) for the period between -9 to +9 days from ovulation

An interphasic difference in dyshoric mood was most pronounced for "lack of self sufficiency" which reached its peak premenstrually shortly after the peak of the hunger rating ($p = .036$ between phases, in the total group). Correlations of mood with calorie intake, and in particular with consumption of fat computed separately for disturbed and nondisturbed eaters revealed significant positive relationships between aggression and food intake during the mid luteal phase in the disturbed group and a negative one in the early follicular phase in the nondisturbed group.

2. Menstrual cycle and hormones: Ovulation tended to occur slightly later (day 17, 4 vs. day 15,2, not significant) and corpus luteum insufficiency (CLI: luteal phase < 10 days and/or P_{max} < 10 ng/ml) was observed more frequently (10 out of 18) in disturbed than in nondisturbed eaters (3 out of 20, X^2 = 6,92, p < .01). Total and early follicular E_2 and P max tended to be lower in disturbed than in non-disturbed eaters but the difference did not reach statistical significance. Peak values of P and total and average luteal E_2 levels tended to be slightly lower in disinhibited than in restraint eaters as defined according to questionnaire subscales of the three factor eating questionnaire (p < .10, p < .10, and p < .05 respectively)

DISCUSSION

The fact that hunger in the total group and calorie intake in disturbed eaters is most pronounced in the mid luteal phase would suggest that desire of eating is associated with an increase in progesterone (as observed in a study by Schweiger et al., 1989 by a correlation between calorie intake and P). However, correlations computed between mid luteal calorie intake and Γ max in our study are around zero indicating that thresholds of P levels required for eliciting appetite or feeding may be interindividually different.

An additional role, however, seems to be taken by follicular E_2 which seems to predict high premenstrual calorie intake in nondisturbed eaters ($r = +.52$, $p < .05$), an observation confirming results by Schweiger et al. (1989), whereas in disturbed eaters rather a high consumption of food (in particular of carbohydrates) in the follicular phase seems to be a prerequisite for development of high ovulatory and luteal values of E_2 ($r = .49$ and .54, $p < .05$ respectively) as claimed by studies on bulimia and starvation (Pirke et al. 1987; Fichter & Pirke, 1989).

The finding of a higher rate of CLI in disturbed eaters would confirm the theory by Fichter and Pirke (1989), that even nonpathological but continuous irregularities of food intake may lead to disturbances of the cycle which may most probably be explained by an impairment of episodic LH secretion (Pirke et al., 1988) possibly associated with feeding related increases in ß-endorphins. This could be corroborated by our finding of lower LH values in disturbed than in nondisturbed eaters on day 5 of the mc ($p < .05$). Furthermore, the positive relationship between consumption of fat and aggression in disturbed eaters in the mid luteal phase could be explained by higher 16α-hydroxylation of estradiol under the influence of high dietary fat (Lustig et al., 1989) which would reduce the emotionally stabilizing function of E_2 in relation to P. This assumption is also suggested by a positive correlation between E_2 and a relaxed emotional state ($r = .64$, $p < .01$) in the mid luteal phase in disturbed eaters.

In conclusion it may be stated that not only irregular intake of food may be responsible for disturbances in release of reproductive hormons, but that abnormalities in times and levels of E_2 and P may also induce changes in hunger and calorie intake.

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SENSITIVITY TO BENOMYL:
THE BODY TEMPERATURE RHYTHM
AND UTERINE METABOLISM DURING
DECIDUALIZED PSEUDOPREGNANCY

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ABSTRACT

That peroral benomyl administration (60 and 80 mg/kg/day) during decidualized
pseudopregnancy caused reductions in the rhythmometric parameters of the body
temperature rhythm. Additionally, when benomyl was administered at 0700 hr (2
hrs after the dark phase) the uterine weight and protein content were adversely
affected. Hence, benomyl affected the body temperature and uterine deciduoma in
an apparent dose-related manner.

KEY WORDS

Benomyl, decidualized pseudopregnancy, body temperature, uterine protein, rats.

INTRODUCTION

Benomyl (methyl-1-butylcarbamoyl-2-benzimidazole carbamate), an extensively used
ε,ʋ.ᵉᵐⁱᶜ fungicide, is an active ingredient in Benlate. The fungicidal potential
of this compound has been shown to be strongly associated with its anti-mitotic
activity (Hammerschlag and Sisler, 1973; Clemons and Sisler, 1971). In agreement
with this inhibitory action, benomyl displays a binding affinity for fungal
tubulin (Davidse 1973; Ireland et al., 1979), and consequently prevents tubulin
polymerization. Similar formation of benomyl-tubulin complexes and blockage of
polymerization also occurred in mammals (Friedman and Platzer, 1978). The
majority of recent studies on mammals, rats and mice especially, has shown that
benomyl exposure can cause many adverse testicular changes (Carter and Laskey,
1982; Carter et al., 1984; Kavlock et al., 1982 and Linder et al., 1988). Hence,
benomyl exposure could be potentially hazardous to activities embodying mitosis
and microtubular involvement. The events associated with growth and synthesis,
which occur during processes like spermatogenesis and fetal development, fall
under this heading. As such, the present study was designed to determine the
anti-mitotic and toxic influence of benomyl during deciduoma formation which
characterizes decidualized pseudopregnancy. This decidual reaction encompasses
the proliferation of the uterine tissue which develops as a result of hyperplasia,
hyperplasia, hypertrophy and the differentiation of stromal cells (DeFeo, 1963).

MATERIALS AND METHODS

Mature, female Sprague-Dawley Rats (220-260 g) obtained from the Holtzman Company

(Madison, WI) were housed separately and maintained under constant environmental
conditions of photoperiod (12L:12D with lights on at 0500 hr) and temperature (21
± 2°C). Rats displaying at least two consecutive estrous cycles monitored by
vaginal lavages, were employed to test the sensitivity to benomyl, administered
in the early morning. Decidualized pseudopregnancy (DPG) was induced by
mechanical cervical stimulation (cs) during protestrus and estrus. This was
followed by laparotomy and the surgical decidual induction of the uterine horns
by wound trauma on day 4 (0900-1400 hr) of persistent leucocytes after cs. The
surgical intra-abdominal insertion of radio transmitters (Model FH from the Mini-
Mitter Company, Sunriver, Oregon) was performed simultaneously. Rats in two
different groups received daily doses (60 and 80 mg/kg/day) of benomyl (technical
grade, 99% purity from Chem Service, West Chester, PA) in corn oil by oral
gavage. Benomyl was administered on days 7-9 DPG at 0700 hr (2 hrs after the
dark phase). Wireless biotelemetry, in conjunction with the Dataquest
computerized system, were employed for continuous acquisition of the body
temperature during days 10-12 DPG. Uterine protein content was assayed by
standard procedure (Lowry et al., 1951) and uterine wet weights were measured at
1100 hr (mid-light) for the 0700 hr benomyl-treated rats.

RESULTS

Table 1. Effect of Benomyl on Rhythmometric Parameters of the Body
          Temperature Rhythm

Group	Mesor	COSINOR Amplitude	Acrophase
Control	37.79**	0.528	10.64
60*	37.50	0.393	12.83
80*	37.73	0.352	15.52

*Represents benomyl in mg/kg/day for 3 days.
**Each reading is the sum of 3 days results on 5 animals.

TABLE 2. Effect of Benomyl on the Body Weight and Uterine Deciduoma

Parameter	0	BENOMYL 60*	80*
Body wt (g)	249**	200	203
Uterine wt (g)	1.53**	1.53	1.23
Uterine Protein (mg/ml)	2.13**	1.78	1.55

*Benomyl dose in mg/kg/day x 3
**Each point is the mean for 5 rats

Generally, the results demonstrate that benomyl administered at 0700 hr had a
pronounced effect on the body temperature rhythm. Both the mesor (37.50 and
37.73) and amplitude (0.393 and 0.352) results following treatment with 60 and
80 mg/kg/day benomyl respectively, were numerically reduced under control values
(Table 1). Also the acrophase of the control rats lagged behind the treated
rats. Additionally, uterine weight and protein, plus the body weights, were all
reduced following benomyl exposure (Table 2). Hence, these data suggest that
benomyl-related toxicity during decidualized pseudopregnancy is apparently dose
related.

ACKNOWLEDGEMENT

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EVALUATION OF INDIVIDUAL AND TEMPORAL PATTERNS IN GASTRIC pH RESPONSE TO RANITIDINE GIVEN AS MULTIPLE BOLUSES OR CONSTANT-RATE IV INFUSIONS OVER 24 HOURS

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ABSTRACT

This investigation focused on individual and temporal differences in response to ranitidine when given over 24 hr as a constant-rate infusion or multiple dose boluses. Greater efficacy was observed with the constant-rate infusions in the responders when compared to the corresponding multiple dose bolus regimens; however, the poor responders showed no differences based on the mode of administration.

KEYWORDS

Ranitidine, circadian rhythm, gastric pH, individual differences

INTRODUCTION

Ranitidine, an H2-receptor antagonist, reduces gastric acid secretion and thus, is an effective means of managing peptic ulcer disease (Strum, 1983; Berardi et al, 1988). A circadian rhythm in gastric acid secretion has been described in healthy subjects. In normal and ulcer patients with a routine of diurnal activity and nocturnal rest, the rhythm is characterized by high unstimulated acid secretion in the evening and low secretion rates in the morning (Moore and Halberg, 1986). Due to the circadian rhythm associated with gastric acid secretion, the timing (morning verses evening of once daily dosing or unequally divided daily dosing) of ranitidine in the treatment or prevention of stress-induced ulcer might prove advantageous. The purpose of this investigation was two-fold: 1) to determine the relative efficacy of equally divided daily bolused doses of ranitidine as a function of time of day and 2) to compare the efficacy of ranitidine (150mg or 200mg) when given as equally divided bolused doses to an equivalent constant rate infusion. In order to address these questions, we investigated a group of participants with healed ulcer disease to whom different doses of ranitidine were administered as constant-rate infusions or multiple dose boluses during separate 24 hr spans.

METHODS

Male subjects, 26-65 yrs of age, with history of duodenal ulcer and diurnal activity alternating with nocturnal sleep were recruited. Each participant was screened medically, which included endoscopic examination to rule out active ulcer disease. Subjects with Zollinger-Ellison syndrome were excluded.

Subjects provided written consent; the study was approved by the Institutional Review Board of the University of Utah.

Patients received a total of 5 treatments, each by intravenous route during 5 separate 24-hr study sessions. Five of the treatments: 1) placebo solution given as a bolus at 0800 and 2000, 2) 50mg ranitidine boluses at 0800, 1600, and 0000, 3) 100mg ranitidine boluses at 0800 and 2000, 4) ranitidine as a 10 mg/hr constant-rate infusion for 24 hr, and 5) ranitidine as a 6.25 mg/hr constant-rate infusion. The constant-rate infusion was delivered from a hanging bag using a mechanical pump. Accuracy of the drug delivery rate was checked hourly by noting bag volume. Subjects were fasted for 8 hrs before and throughout the 24-hr sessions; however, water was allowed ad libitum. No medications were used by the subjects for at least 48 hrs before the start and during each treatment session.

Another publication by members of our group has compared the effectiveness of the bolus versus constant-rate dosing of ranitidine from a conventional, nonchronobiological perspective (Sanders, et al, 1989). This report pertains specifically to the findings for the bolus ranitidine doses in terms of day-night and individual differences in the effect on gastric acidity. In addition, comparisons between the intermittent bolus ranitidine regimens and their corresponding constant-rate infusions are made with respect to the individual differences.

Throughout each treatment session, gastric pH was monitored using a digital recording system (Synetics Medical, USA) at 4-second intervals following methods described elsewhere (White, et al, 1990). Participants reported to the Drug Research Center between 0630 and 0830 before breakfast. The data for pH were converted to hourly mean and median values for each participant. The area under the pH-time curve above pH 4 (pH > 4 was defined as the clinical goal) was calculated using the trapezoidal rule and normalized for the length of the dosing interval. The data were analyzed by analysis of variance or paired t-test for the factors of treatment, subjects and time of day. Additional statistical evaluations were conducted using the Newman-Keuls test.

RESULTS

Of the 15 subjects recruited into the study, only the data of 12 were used for analyzing the effect of ranitidine on gastric acidity. One of the excluded subjects exhibited an abnormal inverse dose-response relationship for ranitidine. Another had a pH > 5 for at least 80% of the time during the 24-hr placebo session. The third subject failed to complete the 6.25 mg/hr regimen.

As previously reported (White et al, 1990) the duration of time during the 24-hr constant rate infusion of 6.25 mg/hr ranitidine that gastric pH was 4 or greater differed between subjects. It was arbitrarily decided that those exhibiting a pH < 4 for less than 16 hr while infused with the low dose of rantidine would be regarded as poor responders. Participants having a pH > 4 for at least 16 hr with this dose were regarded as responders. Eight of the subjects were classified as responders, while 4 were poor responders. This classification was continued in this paper. The poor responders exhibited a distinct high-amplitude circadian rhythm in gastric pH with placebo and both constant-rate infusions of ranitidine. The responders exhibited no circadian rhythmicity in gastric pH when treated with either of the constant-rate infusions and exhibited a higher gastric pH over the 24-hr.

The AUC > pH 4 revealed day-night and individual differences with the 50 mg bolus rantidine dosing regimen (Table 1). Although not statistically significant, the AUC > pH 4 following the initial (0800) 50 mg bolus of ranitidine in both responders and poor responders was 50% greater than that from the subsequent 1600 and 0000 boluses. Overall, AUC > pH 4 was 2-fold greater

Table 1: Area Under the pH-time curve above pH 4 for the 6.25 mg/hr constant-rate infusion and the 50mg (three times daily) multiple dosing regimen expressed as a function of time of administration and 24 hr total.

	0800	1600	2400	24 hr Total
Poor Responders				
50 mg tid	6.7 ± 5.1	4.1 ± 2.7	4.7 ± 2.4	15.5 ± 9.8
6.25 mg/hr	7.7 ± 8.7	2.7 ± 1.9	11.7 ± 12.5	22.0 ± 11.5
Responders				
50 mg tid	12.5 ± 3.7	8.4 ± 5.4	9.5 ± 3.0	30.4 ± 10 8
6.25 mg/hr	19.0 ± 4.8+	13.2 ± 6.1+	15.0 ± 5.7+	47.2 ± 14.3*
All Patients				
50 mg tid	10.4 ± 5.0	6.8 ± 5.0	7.8 ± 3.6	25.0 ± 12.5
6.25 mg/hr	14.9 ± 8.3	9.4 ± 7.2	13.8 ± 8.3	38.1 ± 17.9*

*significant differences detected between the 24 hr total AUC > pH 4 for the 6.25 mg/hr and the 50 multiple dose ranitidine regimens. p < 0.05.
+significant difference between the 6.25 mg/hr and 50 mg multiple dose ranitidine regimens, p<0.05.

Table 2: Area under the pH-time curve above pH 4 for the 10 mg/hr constant-rate infusion and the 100mg (twice daily) multiple dose regimen expressed as a function of time of administration and 24 hr total.

	0800	2000	24 hr total
Poor Responders			
100 mg bid	17.1 ± 6.9	8.60 ± 10.4	25.7 ± 17.3
10 mg/hr	19.5 ± 10.0	21.0 ± 2.4	40.5 ± 11.3
Responders			
100 mg bid	20.5 ± 5.1	20.7 ± 7.4	41.2 ± 11.5
10 mg/hr	33.4 ± 5.4*	32.6 ± 6.4*	66.0 ± 11.4*
All Patients			
100 mg bid	19.6 ± 5.5	17.4 ± 9.6	37.0 ± 14.3
10 mg/hr	29.6 ± 9.1*	29.4 ± 7.7*	59.0 ± 16.1*

*significant differences between the 100 mg multiple dose ranitidine regimen and the 10 mg/hr constant rate ranitidine infusion, p < 0.05.

for responders versus poor responders during each of these 8 hr intervals. Bolusing of 100 mg of ranitidine at 12 hr intervals was associated with neither temporal or responder versus poor responder differences in AUC > pH 4 (Table 2).

The AUC > pH 4 did not differ over the 24 hr between the comparable multiple dose bolus regimens and the constant rate infusions (50 mg tid vs 6.25 mg/hr and 100 mg bid vs 10 mg/hr) for poor responders (Tables 1 & 2). However, in the responders, the constant rate ranitidine infusions produced significantly larger AUC > pH 4, 47.2 ± 14.3 (X ± SD) and 66.0 ± 11.4 for the 6.25 and 10.0 mg/hr treatments, versus 30.5 ± 10.8 and 41.2 ± 11.5 for the every 8 hr regimen of 50 mg ranitidine and every 12 hr bolus of 100 mg of ranitidine, respectively (Tables 1 & 2).

DISCUSSION

Previous studies have shown that gastric acidity vary as a circadian rhythm in both untreated ulcer patients and normal controls with the highest gastric acid secretion in the late afternoon and early evening (Moore and Halberg, 1986). The responders in this study did not exhibit circadian rhythmicity under placebo conditions or with either constant-rate ranitidine infusion. However, the poor responders exhibited a high-amplitude circadian variation under placebo and constant rate infusions treatments. This observation suggests individuality in gastric pH patterns, which constitutes an important consideration in the clinical treatment of ulcer disease.

No differences between the infusion and the corresponding multiple dose regimen were evident for the poor responders. However, in responders the constant-rate infusion had greater efficacy, expressed as a function of AUC > pH 4, when compared to its corresponding multiple dose regimen. No significant differences were found between the 0800 and 2000 100 mg dose of ranitidine for both responders and poor responders; however, the poor responders showed a trend for decreased efficacy with the evening dose of ranitidine as compared to the morning 100mg dose. With the 50 mg ranitidine dose bolused three times daily, both the responders and poor responders showed greater efficacy with the initial 50 mg dose of ranitidine as compared to the 1600 and 0000 doses.

While not always statistically significant, the poor responders exhibited decreased efficacy for each ranitidine treatment. This suggests that a segment of the population may require additional amounts of parenterally administered H2 antagonist medication in the late afternoon and early evening; independent of the administration schedule (constant-rate infusion or multiple dose boluses). It may well be that a programmed-in-time administration of parenteral medication with an increase in dose later in the day will be optimal in the management ulcer crises and/or averting stress induced ulcer in hospitalized patients. These observations require further investigation.

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CIRCADIAN VARIATION OF THE EFFECT OF ASPIRIN ON GASTRIC POTENTIAL DIFFERENCE

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ABSTRACT

The present study was designed to compare the morning and the evening response of gastric potential difference (PD) to aspirin administration in healthy volunteers. The results demonstrate that despite lower pH values in the evening, the irritating effect of aspirin on gastric PD is less pronounced in the evening compared with the morning.

KEYWORDS

circadian variation, transmural gastric potential difference, aspirin injury, gastric mucosa integrity

INTRODUCTION

Recently published data suggest a circadian pattern of susceptibility of gastric mucosa to aspirin induced irritation. In rat, it has been demonstrated that acidified aspirin caused significantly more gastric mucosal lesions when administered in the light phase compared with the dark phase (Olson et al., 1986). In man, a significantly greater mucosal damage induced by aspirin was observed endoscopically after oral administration in the morning compared with that in the evening (Moore and Goo, 1987). The role of circadian variation of transmural gastric potential difference (PD), a sensitive marker of gastric mucosa integrity (Davenport et al., 1964) has not been evaluated yet. The aim of the present study was to assess the reaction of gastric PD to aspirin in the morning and in the evening, each after a 12 hour fasting period in healthy subjects.

MATERIALS AND METHODS

16 healthy, diurnally active volunteers (8 men, 8 woman) with a mean age of 25 (20-30) years participated in the study. PD

response to aspirin has been evaluated by 2 measurements with an interval of 1 week performed in a randomized order at 8 AM and 8 PM, each after a 12 hour fasting period. Prior to the fasting period 1000 ml of a nutrient solution (Fresubin[R], Fresenius AG, FRG) were administered. During the 12 hour fasting time, subjects were allowed only ad libitum tap water until 3 hours before PD measurement.

The potential difference across the stomach wall was measured continuously by means of a flowing intragastric NaCl electrode (Read et al., 1979) with an isotonic saline flow rate of 30 ml/h placed in the antrum under fluoroscopic control and a subcutaneous electrode consisting of a plastic cannula filled with a mixture of 3 M KCl in 3 % agar placed on the volar aspect of the arm. The flowing NaCl electrode, a polyvinyl tube, was attached alongside a Salem sump tube which had been passed nasogastrically. The Salem sump tube was needed for aspiration of gastric content before every test period and for the administration of the test solutions. The 2 electrodes were connected by 3 M KCl agar bridges which were suspended in beakers filled with 3 M KCl. Silver-silverchloride electrodes submerged in the KCl solution were connected via a voltmeter to a recorder.

Basal potential difference was monitored for a period of 30 minutes after instillation of 100 ml isotonic saline. Then, for another 30 minutes, the irritating effect of aspirin on gastric PD was measured after 500 mg aspirin dissolved in 100 ml isotonic saline were administered. Samples of gastric content for the evaluation of intraluminal junction potentials and pH measurements were taken at 5, 15 and 30 minutes during basal period and after aspirin administration.

For statistical analysis of PD, Student's t-test was applied. Wilcoxon's rank sum test was used for the comparison of pH values. PD values are given as mean $\pm$ SEM, pH values are expressed as medians.

RESULTS

Results of PD are demonstrated in Fig. 1. AT 8 AM a significant decrease in PD is observed during the third and 18th minute after aspirin administration compared with corresponding basal values. At 8 PM, only a slight and non significant decrease is observed.
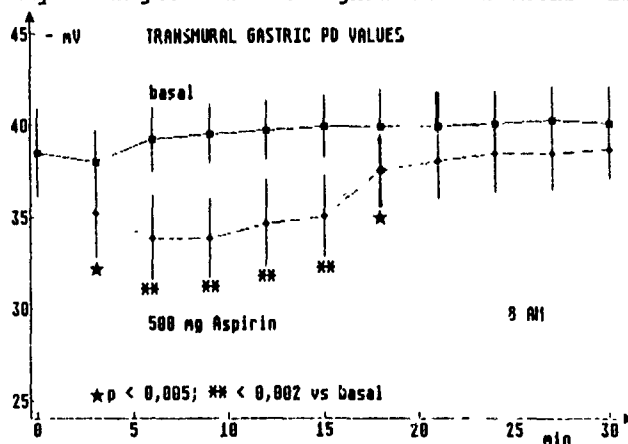
Fig. 1 a: Basal PD and PD after 500 mg aspirin at 8 AM
         * p < 0,005   ** p < 0,002

The mean basal value of PD is lower in the evening (-35,6 ± 1,8 mV), but it is not statistically different from that in the morning (-39,5 ± 1,8 mV). The maximal drop of PD after aspirin administration is significantly (p < 0,001) lower at PM (2,5 ± 0,6 mV) compared with that at 8 AM (7,6 ± 1,2 mV).
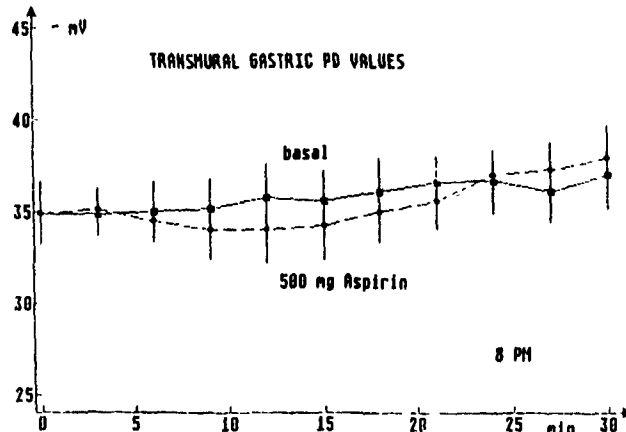
Fig. 1 b: Basal PD and PD after 500 mg aspirin at 8 PM

Similarly, the area under the curve is significantly (p < 0,002) lower in the evening than in the morning (6,4 ± 1,9 vs. 31,4 ± 6,5 mV • min). AT 8 PM, median pH values obtained during the basal period (1,85, 1,80, 1,63) were significantly (p < 0,01) lower than corresponding values (2,20, 2,04, 2,03) at 8 AM.

DISCUSSION

This study demonstrates that the irritating effect of aspirin on gastric potential difference is less pronounced in the evening compared with the morning in healthy subjects. Similar results have recently been observed in the rat (Olson et al., 1986) and in man (Moore and Goo, 1987) by morphological studies of gastric mucosa before and after aspirin administration in the morning and in the evening. Gastric potential difference has been shown to be a sensitive marker of gastric mucosa integrity (Davenport et al., 1964). Stern et al. have recently demonstrated a relationship between the maximal drop of PD and the degree of mucosal lesion induced by an irritant. The higher susceptibility of PD to irritation by aspirin during the morning hours compared to evening hours was not anticipated. Greater evening - time damage was expected because gastric pH is lower in the evening and aspirin induced damage is acid-dependant (Murray et al., 1974).
Greater severity of aspirin - induced damage occuring during the morning hours due to delayed gastric emptying and thus longer exposure to the drug seems improbable. Gastric emptying half-times for the evening meal have been reported to be longer for salids and liquids compared with morning emptying half-times (Goo et al., 1987).
Our data support a role for alteration in mucosal resistance to aspirin induced gastric irritation over the circadian time frame indedepant of the influence of gastric acidity.

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DIFFERENTIAL EFFECT OF SINGLE DIET AND FOOD SELECTION ON TEMPORAL PATTERNS OF FOOD INTAKE AND ARTERIAL AND VENOUS GLUCOSE, INSULIN AND TRYTOPHAN LEVELS IN RATS

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ABSTRACT

Relationships among feeding paradigm (single diet vs food selection) and arterial and venous glucose, insulin and tryptophan levels were studied by measuring the temporal patterns of food intake and plasma parameters during 8 hrs feeding cycles in rats. The feeding paradigms were characterized by opposed carbohydrate and protein intakes that became respectively less accentuated and more accentuated throughout the feeding period, as well as by opposed metabolic responses.

KEY WORDS

Food selection; temporal rhythms; glucose; insulin; tryptophan.

INTRODUCTION

Food made available only at a particular time of the day entrains the circadian rhythms of most mammalian species (Moore-Ede et al., 1982). Meal timing and diet composition are also resulting in marked phase shifts of the circadian rhythms of many biochemical variables (Goetz et al., 1976; Lakatua et al., 1988). Indeed, rats fed a single diet exhibit a circadian rhythm of brain serotonin (Blatter et al., 1986). However, although rats allowed to select protein and energy separately display rhythms in the percentage of energy as protein (%P-E) they select, the rhythm of brain serotonin disappears without any apparent relationship with changes in plasma tryptophan/large neutral amino acids ratio (reflecting tryptophan availability to the brain) (Blatter et al., 1986). The aim of the present work was to study the interaction between diet composition and metabolic events by feeding rats with a single diet of fixed composition or offering a choice of carbohydrate rich and protein rich diets accessible over 8 hrs during the dark cycle.

MATERIAL AND METHODS

One hundred adult male Sprague-Dawley rats (322±6 g) were individually housed at 22±1°C, and adapted to a light/dark (LD) 12:12 cycle (L from 20:00 to 08:00) with free access to water. Half of the animals were fed a 20% casein diet,

and the others were allowed to choose from 0 and 60% casein diets, under 8
hrs time span during the dark cycle (from 08:00-16:00). Casein was interchanged
with corn starch and sucrose so diets were isocaloric. Food was weighed at
2 hourly intervals. After 15 days adaptation, groups of animals were sacrificed
at 08:00, 10:00, 12:00, 14:00 and 16:00 h, and arterial and venous blood was
collected. Plasma glucose was measured by the spectrophotometric method of
Fisher Scientific Ltd (DK, 1200-31/33/36), insulin by a radioimmunoassay
procedure of Bio-Endo Inc., and tryptophan by the fluorometric method of Denckla
and Dewey (1967).

RESULTS AND DISCUSSION

As shown in Table 1, with both feeding paradigms, rats showed similar temporal
patterns of energy, carbohydrate and protein intakes with a peak at the
beginning and a trough at the end of the feeding period. However, in rats
offered a choice of 0% and 60% casein diets part of fractional (from 08:00-
10:00, and 10:00-12:00 h) as well as the cumulative intake of carbohydrate
were significantly lower, whereas all fractional and the cumulative intakes
of energy and protein were found significantly higher than those of rats fed
the single diet. Throughout the feeding period, these differences between
single diet and selection became less accentuated in the case of carbohydrate
intake, but more accentuated in the case of energy and protein intake. Rats
offered a choice of 0 and 60% casein diets thus consumed a larger %P-E
(34.6±0.38% vs 20% for single diet) and a lower percentage of energy as
carbohydrate (%CHO-E) (43.0±0.39% vs 58% for single diet) than single diet
fed rats. The value obtained for %P-E corresponds closely to the proportion
of protein consumed in previous self-selection studies (Musten et al., 1974;
Johnson et al., 1978). The present data extend these observations by
demonstrating that the protein intake regulation using a self-selection paradigm
leads to a significantly higher %P-E than the amount usually fixed in single
diet employed in research. Paradoxically, rats fed a choice of diets had a
significantly higher (p<0.05) daily water intake (28.5±0.95 ml) but a lower
(p<0.01) growth rate (3.57±0.20 g/day) than those found w.th single diet
(25.6±0.74 ml; 4.80±0.16 g/day). On the other hand, feeding with single diet
gave rise to characteristic rhythms of arterial and venous glucose, insulin
and tryp')phan levels, which differed from those found in rats offered a choice
of diets. With diet selection the arterio-venous differences (ΔAV) of glucose
were suggestive of a higher glucose utilization at the beginning of the feeding
period (3.39±0.58), after 2 hrs feeding (3.44±0.10) and at the end of the
feeding period (2.56±0.80), in comparison to single diet (beginning: 2.63±0.48;
2 hrs: 2.42±0.74; end: 1.23±0.41). Moreover, the rhythms of the ΔAV of plasma
insulin and tryptophan, and of arterial and venous tryptophan levels in rats
fed a single diet or a diet choice were in opposite directions. In rats offered
a choice of diets, a significant positive correlation was found between the
fractional %CHO-E per 2 hrs interval and venous insulin level (r=0.66; p 0.01),
whereas negative correlations between protein intake and Δ insulin (r=0.70;
p<0.005), fractional %CHO-E and Δinsulin (r=0.65; p < 0.01) and %P-E and venous
glucose (r=-0.39; p<0.02) were found, suggesting a role of glucose and insulin
in the selection process. Such correlations were absent in the case of single
diet as well as between food intake parameters and plasma tryptophan, the latter
also being reported by Blatter et al. (1986). In conclusion, these data indicate
that the regulatory mechanisms for controlling nutrient intake differ according
to the feeding paradigm used. The opposed metabolic responses to the two
feeding paradigms studied suggest that food intake regulation is determined
by the diet composition rather than by the feeding rhythms.

TABLE 1. EFFECT OF SINGLE DIET AND FOOD SELECTION ON TEMPORAL
PATTERN OF FOOD INTAKE[a]

	Total Food Intake (g)	Carbohydrate Intake (g)	Protein Intake (g)
Mean Intake at 10:00			
Single diet	10.32 ± 0.14^a	6.05 ± 0.85^a	2.06 ± 0.03^a
Selection	11.60 ± 0.23^{a}***	5.12 ± 0.13^{a}***	4.00 ± 0.11^{a}***
Mean Intake at 12:00			
Single diet	5.93 ± 0.01^b	3.48 ± 0.06^b	1.19 ± 0.02^b
Selection	6.54 ± 0.17^{b}**	2.88 ± 0.10^{b}***	2.26 ± 0.07^{b}***
Mean Intake at 14:00			
Single diet	4.32 ± 0.10^c	2.53 ± 0.06^c	0.86 ± 0.02^c
Selection	5.45 ± 0.18^{bc}***	2.35 ± 0.09^{bc}	1.94 ± 0.08^{bc}***
Mean Intake at 16:00			
Single diet	3.31 ± 0.10^c	1.94 ± 0.06^d	0.66 ± 0.02^c
Selection	4.52 ± 0.20^{c}***	1.82 ± 0.08^c	1.73 ± 0.09^{c}***
Cumulative Intake			
Single diet	23.88 ± 0.16	14.01 ± 0.09	4.78 ± 0.03
Selection	27.67 ± 0.42***	12.01 ± 0.23***	9.73 ± 0.20***

[a]Results (mean ± S.E.M.) are derived from the total number of observations
from day 3 to 15.
Statistical analysis was done using student's t-test, *means different from
single diet at $p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and a one way ANOVA followed
by a Duncan's test, numbers followed by the same letter are not significantly
different.

TABLE 2. EFFECT OF SINGLE DIET AND FOOD SELECTION ON TEMPORAL
PATTERN OF BLOOD PARAMETERS (mean ± S.E.M.)

	8:00	10:00	12:00	14:00	16:00
Glucose (mmol/L)					
Arterial					
Single diet	14.12 ± 0.73^a	15.20 ± 1.02^a	15.53 ± 0.47^a	14.52 ± 0.43^a	14.34 ± 0.55^a
Selection	14.17 ± 0.75^a	15.93 ± 0.81^a	15.56 ± 0.49^a	15.01 ± 0.52^a	14.32 ± 1.00^a
Venous					
Single diet	11.48 ± 0.30^b	13.39 ± 0.64^a	12.71 ± 0.31^{ab}	12.43 ± 0.25^{ab}	12.78 ± 0.85^{ab}
Selection	11.02 ± 0.25^c	12.82 ± 0.41^{ab}	12.69 ± 0.47^{ab}	13.18 ± 0.42^a	11.80 ± 0.38^{bc}
Insulin (uU/mL)					
Arterial					
Single diet	43.03 ± 4.01^b	49.62 ± 2.19^b	57.18 ± 5.05^{ab}	63.72 ± 7.90^a	49.45 ± 3.65^{ab}
Selection	54.07 ± 5.00^a	48.85 ± 2.68^a	49.95 ± 9.30^a	66.27 ± 6.71^a	54.00 ± 5.64^a
Venous					
Single diet	77.58 ± 5.74^a	64.62 ± 5.45^a	84.90 ± 7.27^a	85.60 ± 9.07^a	75.44 ± 4.27^a
Selection	70.02 ± 3.10^a	85.36 ± 10.62^a	66.87 ± 11.64^a	86.01 ± 6.53^a	82.15 ± 9.44^a
Tryptophan (ug/mL)					
Arterial					
Single diet	18.38 ± 1.38^c	24.07 ± 1.79^{ab}	23.05 ± 1.68^{abc}	25.54 ± 2.14^a	19.42 ± 1.18^{bc}
Selection	18.68 ± 1.90^c	26.15 ± 1.13^{ab}	21.42 ± 1.48^{bc}	21.66 ± 1.96^{bc}	26.84 ± 0.84^{a}**
Venous					
Single diet	16.42 ± 1.27^c	24.27 ± 1.12^{ab}	25.00 ± 1.92^a	22.04 ± 2.00^{ab}	19.12 ± 1.57^{bc}
Selection	21.32 ± 1.95^a	23.00 ± 1.41^a	17.60 ± 2.09^a	21.86 ± 1.50^a	24.00 ± 1.28^{a}*

Statistical analysis as described under Table 1.

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ACKNOWLEDGEMENTS

^{125}I-INSULIN BINDING IN LIVER AND THE INFLUENCE OF INSULIN ON BLOOD GLUCOSE IN CALORICALLY RESTRICTED B6C3F1 MALE MICE

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ABSTRACT

We have shown that insulin sensitivity has a strong circadian
component in mice. Maximum sensitivity occurs during the light span
(inactive phase in nocturnal mice) and this may be due to alterations
in insulin binding or recptor number (or both) at various times of
day. We have also shown that caloric restriction (CR) has profound
effects upon metabolic rates of specific pathways of intermediary
metabolism. In CR mice, primary emphasis of liver metabolism was
shown to be toward the synthesis of glucose. Young (4 month),
middle-aged (12 month) and old (>24 month) B6CC3Fl male mice which
were either ad libitum fed or CR fed (60% of the amount of food that
the ad libitum conterparts consumed) were studied. Food was
presented just prior to the onset of the dark cycle in all cases. At
four hour intervals a drop of blood was taken via tail puncture for
glucose detrermination from 5 CR or ad libitum fed mice of each age
group. Immediately following, they were injected subcutaneously with
0.4 uCi 125I-Insulin (ICN) which was mixed with 6 IU Lilly regular
insulin. After 20 minutes glucose levels were again determined and
then mice were killed by cervical dislocation, liver was taken and
amount of radioactivity bound was determined in a scintillation
counter. In young ad libitum fed or CR mice a well defined circadian
rhythm for insulin binding was present with maximums occuring near
the time of feeding (transition from light to dark). The level of
binding was significantly higher at all times in CR mice. Glucose
levels decreased during the dark due to exogenous insulin in these
young ad libitum fed animals, but not during the mid-light span.
Glucose levels were uneffected in young CR mice. In middle-aged mice
binding maximums occured somewhat later than in young mice, again
with higher levels of binding occuring in CR mice. Glucose responses

were very unpredictable in ad libitum fed mice and glucose levels
did decrease somewhat in CR mice during the mid-dark. In old ad
libitum fed mice insulin binding was much lowere and not
characterized by circadian variation. CR mice had rhythms which were
similar to those seen in mid-aged mice, but levels of binding were
much lower and acrophase was later in the dark span. Binding in old
CR exceeded that in old ad libitum mice. There was little change in
response to exogenous insulin in either case. Meal timing and
nutritional status appear to have pronounced effects on insulin
sensitivity at various times of day (perhaps at the level of insulin
receptor) and may prove to be important in development of improved
insulin therapy of insulin dependent diabetics.

INTRODUCTION

Caloric restriction has long been known to extend maximual achievable
life-span (McCay et al., 1935, and Maeda et al., 1985), and it is
also evident that restriction of caloric intake delays chronic
degenerative disease processes (Walford et al., 1974, and Sarkar et
al., 1982). Moreover, caloric restriction reduces the ability of a
number os well studied carcinogens to induce cancer (Pollard et al.,
1985). The mechanisms by which these substantial changes are induced
remain obscure, but we have found that changes in regulation of basic
metabolic steps which are hormonally (by insuli and glucagon, and
perhaps others) regulated may be involved (Feuers et al., 1989).

We have also recently found, that insulin sensitivity changes with
time of day and maximum reductions in glucose occur at times of peak
glucose levels (Hunter et al., 1989, and Feuers et al., 1989).
Additionally, it was noted that in preliminary studies, radio labeled
insulin binding occurred just prior to these times. It remains
unclear whether this circadian change in insulin sensitivity is due
to changes in insulin receptor concentration (up or down regulation)
or temporal changes in receptor affinity for insulin. We have
undertaken studies to investigate the circadian insulin sensitivity,
and to attempt to determine if CR might express some of its desirable
consequences thorough such a mechanism.

MATERIALS AND METHODS

A detailed description of husbandry procedures and environmental
conditions has been reported elsewhere (Duffy et al., 1989). B6C3F1
male mice which were either 4, 12 or >24 months of age on either ad
libitum or CR diets (60% of the caloric intake of ad libitum mice,
which was nutrient supplimented) since weaning. Food was presented
to CR mice just prior to the onset of the dark span to simulate as
closely as possible feeding conditions in ad libitum mice. On the
day of the experiment, blood was taken via tail puncture for basal
blood glucose determination from 5 CR or ad libitum fed mice of each
age group. Immediately following, they were injected with 0.4uCi
125I-Insulin (ICN) which was mixed with 6 IU Lilly regular insulin.
After 20 minutes glucose levels were again determined, the mice were
killed by cervical dislocation and livers taken. The amount of
radioactivity was determined in a scintillation counter. The process
was repeated every four hours in order to determine circadian
responses.

RESULTS AND DISCUSSION

In YAL mice insulin binding reached maximum levels at 1400 h, which
was just prior to the time of maximum basal blood glucose levels
(Tables 1 and 2). It seems that receptor concentration and/or
affinity for insulin maximizes (up regulation) in anticipation of
feeding at the onset of the dark span. When these mice were given
exogenous insulin, no response was found at 0600 (mid-light) when
binding levels were low relative to maximums at 1400 h, at which time
maximum reductions in glucose occurred. Thus, it appears that
maximum insulin sensitivity occurs when glucose levels are highest in
YAL mice. Binding patterns were similar in YCR mice, but absolute
levels were much higher (Tables 1 and 2). However, exogenous insulin
did not stimulate glucose uptake in these mice. This suggests that
under CR conditions the liver is protected against glucose uptake.
We have previously noted that in CR mice or rats, metabolic changes
occur which favor glucose synthesis and export. This occurs through
a series of circadian interactions of insulin and glucagon (and
perhaps other endocrine systems). Glucose is thus available for
maintenance of increased motor activity (CR mice are more active than
their AL counterparts) and brain function.

TABLE 1. 125I-Insulin Binding in Young, Middle Aged and Old
Calorically Restricted or Ad Libitum Fed B6C3F1 Male Mice at
Different Times of Day.

CPM/GRAM TISSUE

DIET	AGE	0200	0600	1000	1400	1800	2200
AL	Y	---	9200	---	9900	---	5225
	M	6885	6622	6518	7100	8312	6720
	O	3522	2101	2620	2680	2610	4808
CR	Y	5600	9406	11680	12222	10800	7420
	M	9185	9240	11200	10850	15800	8850
	O	6680	4200	4250	5180	5320	4185

AL=ad libitum fed; CR=calorically restricted (60% of ad libitum fed)
Y=young (4 months); M=middle aged (12 months); O=old (>24 months)

Similar results occur in mid-age animals, except that less binding
occurs in mid-age and still less in old mice. CR mice always
demonstrate higher levels of binding (Tables 1 and 2). Additionally,
in mid-age maximum binding occurs at the beginning of the dark span
and is further phase shifted into the dark span in OCR and OAL mice.
Thus, with aging loss of ability to temporally regulate insulin
receptor occurs and this may play a role in moderated ability to
sustain circadian rhythms of blood glucose as was present in younger
animals. Since both CR and AL mice demonstrated phase shifting as a
function of age, CR probably does not interact in offsetting this
aging phenomena.

It has been widely assumed that for insulin to work, it must bind.
Here we demonstrate that in CR mice more binding occurs at all times
of day, but less glucose uptake results. However, it must be noted
that other insulin stimulated cellular events actually are
potentiated (Feuers et al., 1989). Additionally, it is suggested
that CR stimulates mechanisms which protect against glucose uptake.

This observation is consistent with conditions which would be
expected which would help to avoid excess macro-molecular glycation.
Glycation has been set forth as one major theroy of aging.
Importantly, we have shown that in rodents, the early dark span
provides a time of maximal insulin sensitivity. It seems that this
observation along with dietary considerations (and meal timing)
should have clinical implications; especially when dealing with
diabetic patients.

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CIRCADIAN VARIATIONS
OF RADIOINSULIN-RECEPTOR BINDING
IN CALORIC RESTRICTED MICE
AND RELATIONSHIPS TO
CHRONOTHERAPY OF HUMAN INSULIN
DEPENDENT DIABETES

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ABSTRACT

The binding of ^{125}I-Insulin to liver and epididymal fat as well as assay of the
hypoglycemic, peripheral effects of regular insulin was measured at six differ-
ent 4 hour intervals beginning at 0600 in normal ad libitum (ad lib or AL) and
in caloric restricted (CR) B6C3F1/BR male mice and expressed as specific
cpm/Gm wet weight activity. This was done on 4 months old, 1 year old and 25
months old AL and CR mice. The oldest mice showed an expected lower level of
radioinsulin binding especially during the light phase in both liver and espe-
cially fat. Middle age AL livers showed less than half the binding of the
lighter weight CR mice while binding in fat of AL mice was only about 1/4 that
of the slender CR especially during the late light phase. Caloric restricted
animals of all ages typically showed greater insulin binding than AL of similar
ages. Insulin which cannot bind generally cannot work yet AL were unable to
survive lethal hypoglycemic insulin treatment unlike CR perhaps due to inade-
quate adrenergic neural compensation. Sensitivity to exogenous epinephrine
noted by hyperglycemia showed a 3 to 4 fold difference with identical doses
producing lowered glucose elevations in CR mice during the dark period (1830 or
0230) in 3 years old mice. AL "old" mice also showed a significantly higher
mortality at 25 months age associated with hepatic neoplasia. The value of
weight loss and effects of "down-regulation" of receptors by excess adipose
and insulin was recognized and utilization of chronotherapy using insulin to
treat human Type I diabetes (IDDM) has been conducted (by JH). Administration
of 90% of a split-dosage of NPH and regular insulin prior to breakfast and 10%
of the total daily dose of insulin prior to dinner together with proper caloric
restriction of diet and exercise has resulted in a 20% weight loss with a 40%
reduction in insulin requirement. Achievement of euglycemia characterized by
an increased incidence of "hypoglycemic unawareness" and overt hypoglycemia
that is considered by some authorities as "normal" has been noted.

INTRODUCTION

Previous studies of Hunter, Feuers, Tsai and Scheving at the NCTR in 1988 have
indicated that radioiodinated insulin is bound to receptors in fat and liver of
mice in variable concentrations when identical dosages are given at different
times of day (Feuers, et al, in press, 1989). Activity noted as cpm/Gm wet
weight was lowest when 0.5μCi ^{125}I-Insulin was injected at 1030 and a 2 to 3
fold greater binding of the same dose occurred in liver at 1830 and a 16 fold
higher cpm/Gm in fat at 0230 during the dark phase. Circadian variations have
been reported in responses to exogenous insulin noted in hypoglycemia and in
mortality to alloxan which elicits an endogenous insulin release particularly
during the midlight phase (Hunter, et al, 1988). Recent studies on caloric
restricted rats and mice suggest that CR inhibits onset of diabetes observed in
ad libitum fed animals with insulin levels closely correlated to times of
feeding, activity, respiration, etc. (Duffy, et al, 1989). The chief objective
of this study was to measure radioinsulin binding to liver and peripheral
epididymal fat after a 20 minute receptor-binding period in identical ^{125}I-
Insulin binding doses (0.4μCi) given to groups of AL and CR normal mice at 6
different circadian intervals. Mice of three different ages were ultimately
compared at 4 months, 1 year and 25 months of age. Implications for the
chronotherapy of Type I diabetes later became apparent to one insulin-dependent
diabetic (JH) during these ongoing studies.

MATERIALS AND METHODS

Three dozen male B6C3F1 mice of three different age groups (4 months, 12 months
and 25 months) on an ad lib (AL) or caloric restricted (CR) diet (60% of AL
supplement with vitamins and minerals) were light adapted to a 12:12 LD cycle
(light 0600-1800). Six different circadian time intervals were established
for analysis of binding of 0.4μCi/0.15 cc to which 6 U of regular, unmodified
beef insulin was added to moderate and stabilize the uptake and binding
kinetics. ^{125}I-Insulin was injected, sub-cutaneously into groups of 6 mice at
4-hour intervals at 0230, 0630, 1030, 1430, 1830 and 2230. After exactly 20
minutes, a blood sample was taken for glucose reflectometer assay and mice
were sacrificed and 0.5 Gm of the left lobe of liver and left epididymal fat
pad was removed, processed, weighed and cpm/Gm measured in a Packard Auto-
Scintillator Spectrograph standardized for ^{125}I assays. One and 2 way ANOVA
variance analyses and t-tests were utilized for data analysis at NCTR. Subse-
quent studies are presently concerned with assays of the effects of identical
doses of epinephrine and/or glucagon in elevation of blood glucose in both AL
and CR mice. Application of observations on NCTR mice was also employed in
modifications of the chronotherapy of a human Type I-insulin dependent diabetic
using long-duration NPH (N) insulin injected at 0700-0730 (prebreakfast) with
a second, separate dose of rapid-acting regular (R), the dosage of which depend-
ing upon blood glucose self-test analyses in balance with proper work timing,
diet and exercise. A single, predinner dose of insulin (1800-1930) based on
blood glucose level at approximately 10% that of the morning dose has been
essential to accomplish euglycemia. Reduction of body weight by appropriate
caloric restriction and reduction of excessive insulin dosage leading to a
down-regulation of membrane insulin receptors was conducted over a 9 months
period.

Results

Insulin binds to target receptors and activates reactions which lead to a rapid
uptake of the sugar into liver, fat and muscle. These "rapid actions" can result
in hypoglycemia. Insulin also has "slow effects" which support anabolic growth
activities involved in caloric restriction studies (Levine,1982). Oldest mice
showed an expected lower level of radioinsulin target binding especially during
the light phase. Similar results were noted in young mice. Middle age mice

Fig. 1. Circadian Rhythm of Insulin Receptors
Insulin binding to Fat Receptors

cpm\Gm. in 1000's/gm (Thousands)

Time - hrs of day

— Caloric Restricted   — Ad Lib fed mice

8 mice per Time pt.
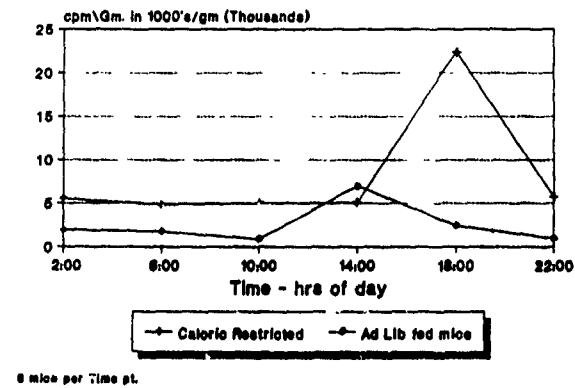
Fig. 2. Circadian Rhythm of Insulin Receptors
Binding to Receptors in the Liver

cpm\Gm. in 1000's/gm (Thousands)

Time - hrs of day

— Caloric Restricted   — Ad Lib fed mice

8 mice per Time pt.
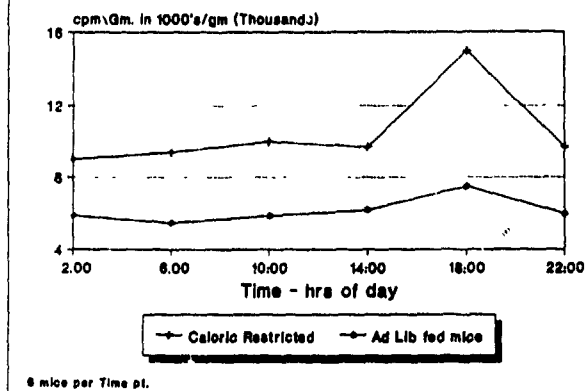
Fig. 3. Blood Glucose after Insulin Saturation
In caloric restricted and 'ad lib' mice

Glucose - mg\dl

Time after Injection

— Caloric Restricted   — Ad Lib fed mice
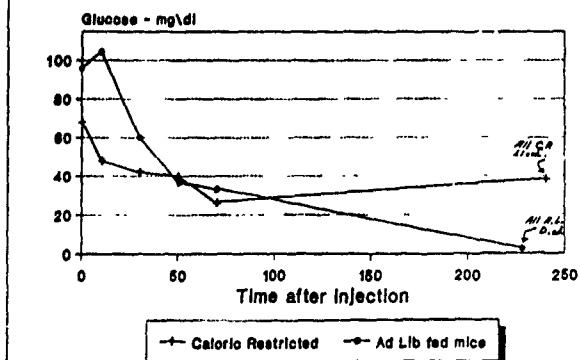
TABLE I. Blood Glucose Changes in Response to Insulin by Human Type
I-IDDM in Representative Samples from Prebreakfast (AM) and
Predinner (PM) Dosages

Date	Insulin -Dose-	A. Basal Glucose		B. Post-Insulin		C. Changes	% Decline	Comments & Ratio of Changes :
		mg\dl	Time	mg\dl	Time	(A-B)	C/A	AM vs PM
5/6/85	5 - R	93	0830	84	0900	9	10	Breakfast
" " "	5 - R	74	1807	52	1837	22	30	Dinner 3.0
5/85	6 - R	120	0800	110	0830	10	8	Breakfast
" "	6 - R	84	1800	62	1830	22	26	Dinner 3.3
2.10.90	10 - R	308	0145	291	0245	17	6	Early AM
2.22.90	58 NR	403	0730	289	0830	114	28 ·	Breakfast

R - Regular Insulin, N - NPH Insulin, NR - "split dose", N&R in separate
doses; Assay by MediSen.·-, Inc.-ExacTech Blood Glucose Sensor.

especially CR showed significantly higher levels of binding. AL livers showed
less than half the binding of the CR mice while binding in fat of AL was only
about 1/4 that of the CR during the late light phase (1830-figs 1+2). CR mice
of all ages typically showed greater insulin binding than AL of similar ages.
Insulin unbound generally cannot work yet AL mice were unable to survive lethal
hypoglycemic insulin (fig. 3) unlike CR due possibly to inadaquate adrenergic
activity. Studies of E chronosensitivity reveals a significant different
(lower) hyperglycemic response to E during the early dark phase vs AL with
enhanced survival. AL mice showed much higher mortality at 25 month associated
with hepatic neoplasia. The value of weight loss (CR) was noted by a Type I
diabetic (JH) and effects of "down regulation" of receptors by excess adipose &
insulin over past 9 month. Achievement of euglycemia is characterized by
occasional "hypoglycemic unawareness" considered "normal" by many medical
authorities. Chronotherapy with insulin involves use of 90% of a split dose (NR)
given at breakfast (0700-0800) when receptors seem to have lowest reactivity plus
10% of the daily net dose given prior to dinner (18- 1930) when receptors or
hypoglycemic response is 3-3.3 fold greater than in morning (Jarrett,1979).
These observations may be of interest.

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CIRCADIAN RHYTHM OF THE LEVEL OF INSULIN, GLUCAGON, GROWTH HORMONE AND PANCREATIC POLYPEPTIDE IN THE BLOOD OF INSULIN DEPENDENT DIABETIC CHILDREN

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ABSTRACT

Diurnal variation in the level of insulin,glucagon,growth hormone
and pancreatic polypeptide were studied in diabetic children.
In the evening hours higher blood level of the investigated
hormones were found.

KEYWORDS

Insulin; glucagon; growth hormone; pancreatic polypeptide;
diurnal variation, blood level, insulin dependent diabetes

INTRODUCTION

The circadian rhythm of the excretion of hormones is the subject
of numerous works which analize that problem both in experiments
on animals and in observations in people / 2,3,4 /. In a lesser
degree do these observations relate to children in whom the course
of various circadian rhythms is different as depending on the
scale of age. The subject of our interest is the circadian rhythm
of insulin, glucagon, growth hormone and pancreatic polypetide
in healthy children and insulin dependent diabetic children in
connection with insulinotherapy applied to them.
Within the range of humoral regulation, particularly in the course
of insulin dependent diabetes, it is glucagon that plays an
essential part as it increases the secretion of insulin and
somatostatin. The introduction of radioimmunological methods made
it possible to obtain closer data for the evaluation of its level
in the blood serum in the course of that diseases. The levels
of glucagon in the blood in diabetic patients are increased /6/.
Closer data on the circadian rhythm of its level are wanting.
In the range humoral regulation attention has been paid to the
pancreatic polypeptide produced in the pancreatic islets /5/.
The mechanism of its action is not accurately known. What is
supposed, too, that it stimulates the secretion of somatostatin
and decreases the secretion of insulin. Its secretion increases

under the influence of hypoglycaemia and of the stimulation of th. nervous system /1,5/. Its circadian rhythm is not known.

MATERIALS AND METHODS

The observations were carried out in 138 children /71 boys and 67 girls/ aged 6-12 years with insulindependent doabetes. The children were receiving monocomponent insulin in 2 daily injections. The level of insulin, growth hormone, glucagon and the pancreatic polypeptide was determined radioimmunolocally. The obrained results were compared with similar clinical observations in healthy children.

RESULTS

The basical secretion rhythm of the growth hormone was similar in healthy childrenand in diabetic children, in the latter, however, higher levels of that hormone have been found. The concentration of the growth hormone in the blood serum was reaching in the night hours higher vakues, statistically significant, than in the day hours /Table 1/.

Table 1. Growth hormone level in the blood

Time	Growth hormone /uJU/	
	Healthy children	Children with I.D.D.M.
8 a.m.	9,8 + 0,3	22,4 + 0,8
11 a.m.	1,8 ∓ 0,2	1,8 ∓ 0,3
2 p.m.	1,2 ∓ 0,1	2,2 ∓ 0,2
5 p.m.	1,3 ∓ 0,1	2,4 ∓ 0,3
8 p.m.	1,2 ∓ 0,2	1,2 ∓ 0,2
3 a.m.	11,2 +0,1	14,9 ∓ 0,6
5 a.m.	13,2 ∓0,4	24,2 ∓ 0,9

The level of insulin in the blood serum has shown in our clinical observations also a markedly variability within the 24-hour day. As it is evident from our table, the level of insulin in the early and late night hours was getting increase as compared with the values noticed during tha day hours /Table 2/.

Table 2. Insulin level in blood

Time	Healthy children	Children with I.D.D.M.
8 a.m.	31,8 + 2,6	16,8 + 2,2
11 a.m.	12,6 ∓ 1,8	9,2 ∓ 2,4
2 p.m.	22,4 + 2,2	11,9 ∓ 1,1
5 p.m.	15,8 + 2,1	7,6 + 3,4
8 p.m.	11,9 ∓ 5,6	5,4 ∓ 3,3
11 p.m.	27,3 ∓ 2,8	15,2 ∓ 2,9
3 a.m.	36,4 ∓ 1,5	27,3 ∓ 3,2
5 a.m.	32,3 ∓ 3,3	25,8 ∓ 4,8

The concentration of glucagon in the blood serum is markedly higher in insulindependent diabetic children. However, both in healthy children and in those with diabetes a distinct daily rhythm with its highest values at 24 o'clock is to observed /Table 3/.

Table 3. Glucagon level in blood /pg/ml/

Time	Healthy children	Children with I.D.D.M.
6 a.m.	348,1 + 81	415,8 + 27
12 a.m.	210,5 + 76	395,2 + 64
6 p.m.	405,6 + 26	705,8 + 36
12 p.m.	728,6 + 12	1116,6 + 12

The concentration of the pancreatic polypeptide, on the other hand, is nit subject to so marked a grow h in children with diabetes as it takes place in the case of glucagon. Only as inconsiderable increase of it is to be stated. Also in these observations one can find, however, daily fluctuations in the blood serum with the highest values in the early night hours /Table 4/.

Table 4. Pancreatic polypeptide level in blood /pg/ml/

Time	Healthy children	Children with I.D.D.M.
6 a.m.	120,8 + 16	180,9 + 12
12 a.m.	115,1 + 26	171,8 + 34
6 p.m.	253,6 + 18	314,2 + 39
12 p.m.	341,8 + 61	498,3 + 67

CONCLUSIONS

The obtained clinical observations, point, in our opinion, at the great sugnificance of the knowledge of the circadian rhythm in the blood serum for the diagnosis and therapeutical procedure in the insulindependent diabetes in childre.

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SECTION 3

CARDIOVASCULAR AGENTS

Chairpersons: B. Lemmer and J. Cambar

RENAL HAEMODYNAMICS CIRCADIAN RHYTHM IN UNANESTHETIZED UNRESTRAINED DOPAMINE INFUSED RATS

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ABSTRACT

The present study reports urinary solutes excretion circadian changes in continuous intraveinous 0,9% NaCl or dopamine infusion in chronic veinous and arterial canulated rat. Such instrumented animals permit to evaluate coninuously during the day the renal haemodynamic changes estimated by inulin and para-amino-hippuric acid (PAH) clearances; Cin represents glomerular filtration rate (GFR) and CPAH renal plasma flow (RPF). In controlled environmental conditions, urine was collected every 4 hours (at 12.00, 16.00, 20.00, 24.00, 04.00 and 08.00) and blood collected at mid-time of urine collected period. Diuresis, GFR and RBF present large circadian variations with a similar night peak in control and dopamine rats.
So, circadian changes in renal haemodynamics and in urinary excretion persist during NaCl and dopamine continuous infusion during many consecutive days in this experimental animal model of unanesthetized unrestrained rat, opening so the way to further investigations in renal chronopharmacology and chronotoxicology.

KEY-WORDS

Circadian rhythms, renal haemodynamics, continuous infusion, chronically canulated rat, dopamine.

INTRODUCTION

Circadian changes in water, electrolytes or solutes excretion have been extensively reported in rodents (COHN, 1970 - ROELFSEMA, 1980 - CAMBAR 1981).
At opposite, evidence of such circadian changes in renal haemodynamics is very poorly documented. We had reported such temporal changes in glomerular filtration rate (GFR), grossly assessed by creatinin clearance (CAMBAR, 1978).
The present study reports circadian changes in renal haemodynamics, e.g. GFR and renal blood flow (RBF), assessed respectively by inulin and PAH clearance estimation in chronic canulated unanaesthetized and unrestrained rats. This experimental model seems to represent the best physiological conditions to study, during consecutive periods, renal haemodynamics and urinary electrolyte excretion. Similar experiments are done in two rat lots receiving 0.9% NaCl or dopamine continuous infusion.

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MATERIALS AND METHODS

Sprague Daley male rats, weighing 200 - 220g, are chronically canulated. Calibrated catheters (Plastimed PE1 and PE2 respectively 0,58 and 0.30mm interior diameter) are placed in femoral artery and in jugular vein.

Animals are placed single in special metabolism cages with separate urine and feces collection and are related to a particular device allowing continuously urinary and blood collect. All experiments are located in a chronobiologic box with constant température (24 - 25°C), atmospheric pression (750 - 770 mmHg) and hygrometry. Moreover, this box presents light automatically controlled conditions 12L / 12D (light period 8h-20h and dark period 20h-8h).

Urines are collected during 6 consecutive 4 hours periods (08.00-12.00, 12.00-16.00, 16.00-20.00, 20.00-00.00, 00.00-04.00 and 04.00-08.00). Blood are collected via femoral artery at mid-time of urine collect period at 10h, 14h, 18h, 22h, 2h et 6h. Dopamine (2,5 ug/kg) or 0,9% NaCl is continuously constantly infused en via jugular vein at 0,67 ml/h.

Diuresis is directly measured; urinary electrolytes and serum and plasma inulin and paraamino hippuric acid (PAH) are assessed by classical spectrophotometric method. GFR and RBF can be estimated by inulin and PAH clearance calculation. Clearances and diuresis are expressed in ml/mn for each 4 hours period.

RESULTS

a) <u>Diuresis circadian variations</u> . In 0,9% NaCl infused rats, 24 hours diuresis is 62,25 ml. A circadian variation in water excretion can be detected with a night maximum; 20.00 - 08.00 diuresis represents 60,11% total diuresis with an acrophase between 00.00 and 04.00 (22,41% total 24 hours diuresis). In dopamine infused rats, 24 hours diuresis is 92,82ml with a 49,11% increase. Circadian variation in diuresis remains with a night maximum (65.99%) and an acrophase between 00.00 and 04.00 (24.78%).

b) <u>Inulin clearance</u> (e.g. GFR) <u>circadian variations</u> (figure 1). In 0,9% NaCl infused rats, circadian variation in inulin clairance is similar with the same night increase (59.13%) and acrophase between 00.00 and 04.00 (22.21%). In dopamine infused rats, inulin clearance mesor is increased (+ 26.73%). GFR circadian variations remain with a night maximum (58.93%) and an acrophase between 00.00 and 04.00 (22.32%).

c) <u>PAH clearance</u> (e.g. RBF) <u>circadian variations</u> (figure2). Likewise, in 0,9% NaCl infused rats, similar circadian variations in PAH clairance can be evidenced with the same night maximum (58.88%) and acrophase between 00.00 and 04.00 (22.20%). In dopamine infused rats, PAH clearance is also increased (+10.43 %). RBF circadian variations remain with a night maximum (58.92%) and an acrophase between 00.00 and 04.00 (22.19%).

d) In these dopamine infused rats, <u>GFR and RBF present similar chronograms</u> with a similar peak between 20.00 and 04.00 (GFR 2,0 + 0,4 ml/mn, RBF 4,8 + 0,5 ml/mn) and a decrease between 12.00 and 20.00 (GFR 1,1 + 0,2 ml/mn, RBF 2,8 + 0,3 ml/mn).

DISCUSSION

If it has been extensively evidenced temporal changes in solutes excretion in experiment animals, rare reports have been done in renal haemodynamics (Cambar et al., 1981). Chronic veinous and arterial canulated rats permit to evaluate coninuously during all the day the renal haemodynamic changes estimated by inulin and para-amino-hippuric acid (PAH) clearances. These instrumented animals allow to compare in 6 consecutive 4 hours periods changes in glomerular filtration rate (GFR) and in renal blood flow (RBF), a better approach than endogeneous creatinin clearance already reported (Cambar et al, 1978). Significant circadian changes in renal haemodynanics can be so evidenced with a nightly increase and a acrophase between 00.00 and 04.00.
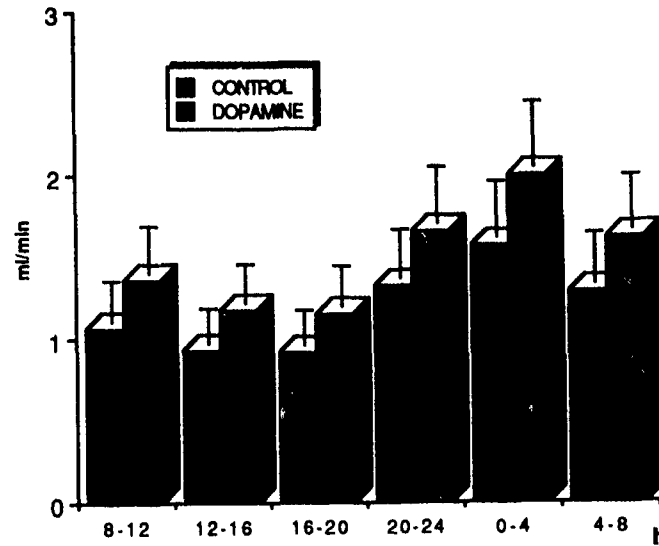
Fig. 1. Circadian variations in inulin clearance (glomerular filtration rate) in unanesthetized unrestrained rat in six consecutive periods.
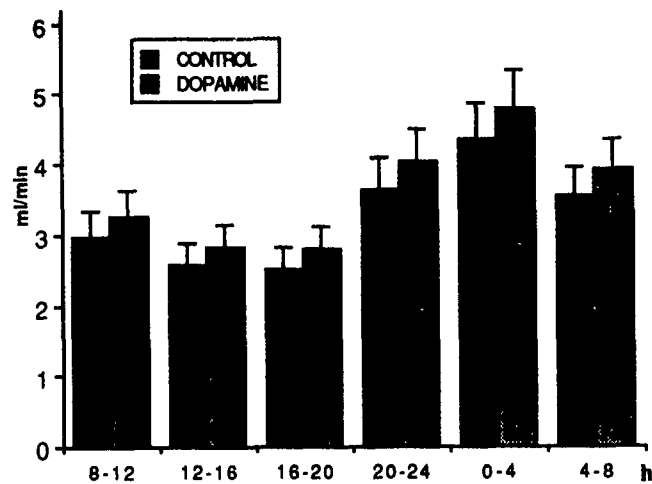
Fig. 2. Circadian variations in PAH clearance (renal blood flow) in unanesthetized unrestrained rat. sodium and chloride reabsorption in dog.

Circadian variations in urinary electrolytes or nitrogen compounds have been evidenced to be unchanged in animals receiving intraveinous or intragastric continuous solutes infusion (Poulis and Roelfsema, 1989 - Roelfsema and van der Heide, 1980, 1982). The present study reports similar data; in both NaCl and dopamine continuous infused unanesthetized unrestrained rats, renal haemodynamics and solute urinary excretion rhythms can persist during many consecutive days.

Such experimental device opens the way to further investigations in renal chronopharmacology and chronotoxicology.

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CIRCAMENSUAL CHANGES IN RENAL HAEMODYNAMICS AND TUBULAR FUNCTIONS IN UNANESTHETIZED DOG

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ABSTRACT

If it has been extensively evidenced circadian changes in electrolytes and solutes excretion, rarely have been reported circannual changes in urinary excretion and renal haemodynamics in laboratory animals, as rodents or dogs. The aim of this study is to evidence circannual (circamensual) changes in renal haemodynamics and tubular functions in unanesthetized female Beagle. Female dogs are prepared with chronic venous and arterial canulation for IV infusion (NaCl, inulin and PAH) and blood collection. Urine is collected in special dog metabolism cage. Such chronic devices permit to evaluate, in the same animal, at different months in two consecutive years, 12 different parameters of renal haemodynamics and tubular functions. For the different renal parameters, it can be evidenced a significant circannual rhythm: changes in haemodynamics are very weak, but changes in tubular functions are large. For example, changes in Na and Cl excretion are highest in winter and similar for both electrolytes. A remarkable opposite curve "in mirror" for Na and Cl reabsorption can be noted with a dramatic decrease in november. Such original chronobiological data in dog will confirm those yet described in hamster and marmot. Such data show that we must in the future take in count as well circadian changes as circannual ones to understand the renal physiology, but also the renal pharmaco-toxicology.

KEY-WORDS

Circannual, circamensual, renal haemodynamics, tubular functions, unanesthetized dogs.

INTRODUCTION

For the previous studies of Vogel in 1854, numerous investigations have reported existence of circadian changes in urinary solutes excretion in man and more recently in laboratory animals, as rodents or dogs. Ultradian and circadian urinary excretion variations have been reported in dog (Gordon and Lavie, 1982).
If it has been extensively evidenced circadian changes in solutes excretion, rare reports in circadian changes have been done in renal haemodynamics of these animals.

Seasonal or circamensual changes in urinary excretion have been not yet reported in dog as it has been done in rat, hamster or desertic rodents.
The aim of this study is to evidence circamensual changes in renal haemodynamics and tubular functions in unanesthetized female Beagle frequently used for physiological renal experiments every month during two consecutive years.

MATERIALS AND METHODS

Female Beagle dogs, weighing 12 ± 2 kilograms, are prepared with chronic venous and arterial canulation for IV infusion (NaCl, inulin and PAH) and blood collection. Urine is collected in special dog metabolism cage. Such chronic devices permit to evaluate at different months in the year numerous parameters in the same animals: (1) the renal haemodynamics; inulin and PAH clearances e.g. glomerular filtration rate (GFR) and renal blood flow (RBF): (2) the tubular functions; diuresis, Na, K, Cl excretion, Na and Cl reabsorption rate and clearance, free water and osmotic clearances. These experiments were done during two consecutive years.

RESULTS

For all parameters studied monthly, a fair reproducibility in results can be noted in these two consecutive years.
For all 12 parameters, circannual (or circamensual) variations (C.V.) can be detected with more or less large amplitude.
C.V. in diuresis (0,54ml/mn/kg mesor) present a very weak amplitude with a minimum in summer and a maximum in winter.
C.V. in haemodynamics, e.g. glomerular filtration and renal blood flow, present also very tiny amplitude with a minimum in february.
In opposite, changes in tubular functions are large. Sodium and chloride excretion present similar C.V. with an increase in winter months (figure 1). Potassium excretion is increased in november - december, but maximum is noted in september. Likewise, sodium and chloride reabsorption describes large C.V. with a dramatic decrease in november (figure 2). But, sodium and chloride clearance shows a very significant increase in winter, augmentation fairly "in mirror" with the decrease in both solute reabsorption. Moreover, free water clearance is highest in november and osmotic clearance highest in february and smallest in july.

DISCUSSION

If numerous investigations have reported existence of circadian changes in urinary solutes excretion in man and in rodents, ultradian and circadian excretion variations have been rarely reported in dog (Gordon and Lavie, 1982). Moreover, if it has been extensively evidenced temporal changes in solutes excretion, rare reports have been done in renal hemodynamics of experiment animals (Cambar et al., 1979). Finally, seasonal or circamensual changes in urinary excretion have been not yet reported in dog, as it has been done in rodents.
This study evidences, in unanesthetized dogs, such circannual changes (with measures in all months in two consecutive years) in 12 renal parameters, e.g. renal haemodynamics and tubular functions. Such original chronobiological data in dog will confirm those yet described in hamster (Haberey et al., 1967) and in marmot (Zatzman and South, 1981).
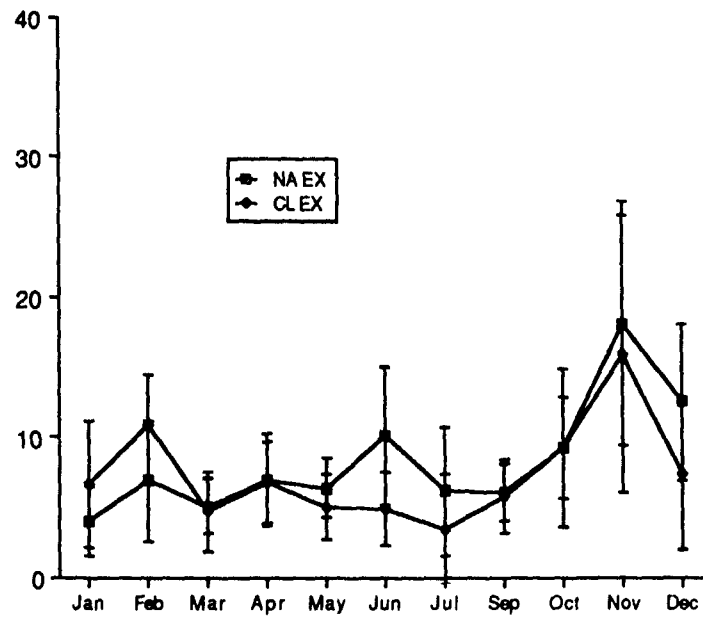
Fig. 1. Circamensual variations of sodium and chloride excretion in dog.
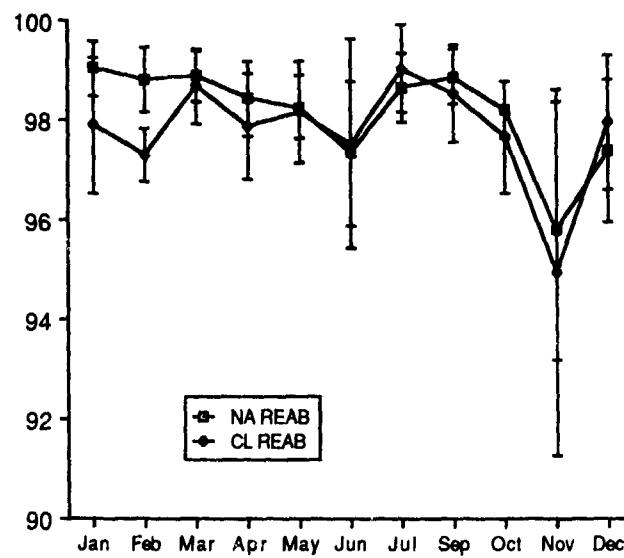
Fig. 2. Circamensual variations of sodium and chloride reabsorption in dog.

Such data show that we must in the future take in count as well circadian changes as circannual ones to understand the renal physiology but also the renal pharmaco-toxicology (Cal et al., 1985).

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COMPARATIVE STUDY IN PLASMA
ATRIAL NATRIURETIC FACTOR (ANF)
CIRCADIAN VARIATIONS
IN NORMOTENSIVE AND
HᵛPERTENSIVE RAT

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ABSTRACT

The present study purposes to evidence eventual circadian changes in some circulating renin-angiotensin system (RAS) components, as hormones or enzymes, in normotensive rat (NT) and to compare them to those of hypertensive rat (SHR strain). 72 NT and 36 SHR rats are housed in controlled environmental conditions with a L/D 12:12 illumination schedule light. At six consecutive times (08.00- 14.00 - 20.00 - 02.00 - 08.00 and 14.00), lots of rats were killed by decapitation and blood samples collected Urines are also collected during consecutive 6 hours periods. Renin activity, angiotensin II, aldosterone, converting enzyme, atrial natriuretic factor (ANF), corticosterone and electrolytes were measured in plasma. Moreover, urinary electrolytes and aldosterone are dosed. Cosinor analysis can detect a circadian rhythm and permits to quantify chronobiological variables for each considered parameter. In normotensive rats, all RAS components present significant circadian changes, with an acrophase at the beginning of the dark period. In SHR rats, such similar circadian rhythms can also be detected for all components except for ANF. ANF presents a bimodal variation with two peaks in the middle of the light and of the dark period. It can be so carefully advanced that ANF circadian rhythm could be a marker to evidence hypertension in rat. Such study will permit also to better understand the structure of the RAS, as well the circulating one as the recently discovered local tissue ones.

KEY WORDS

Circadian variations, hypertension, hypertensive rat, atrial natriuretic factor, renin-angiotensin-aldosterone system.

INTRODUCTION

The renin-angiotensin-aldosterone system (RAS) is the main system involved in the regulation of blood pressure and the maintain of electrolytes and volume homeostasis For two decades, RAS plays a very important place in physiology and pharmacology of hypertension Since that time, many studies have well established the existence of circadian rhythms in systolic and diastolic pressure in healthy subjects as well as in hypertensive patients

A good knowledge of the chronobiology of blood pressure permits to exactly define hypertension existence and severity Since blood pressure shows so striking circadian changes, it is logical to look for similar changes in numerous factors controlling it. Among them, RAS components role can be advanced. Its structure and functions are of great complexicity; its main target organs are cardiovascular system, kidneys and also adrenal glands Recently, the atrial natriuretic factor (ANF), discovered in heart, interferes with renin and aldosterone, and seems to play also a role in sodium and blood pressure regulation by a striking opposition with RAS. Moreover, the existence of a local angiotensin production, discovered within 12 different tissues, makes it much more difficult to understand the regulating processes of the whole system (Dzau, 1987). There is evidence for the existence of circadian rhythms in circulating RAS, in mammals, as well in man (Donckier et al.,1986 - Sumioki et al., 1989 - Yoshino et al., 1989) as in rat (De Forrest et al., 1979 - Hilfenhaus, 1976 - Laval,1988 - Rodriguez-Sargent, 1982) The aim of the present study is a chronobiological approach of most of the circulating RAS components, namely ANF. Plasma renin, aldosterone, corticosterone, angiotensin II, converting enzyme and ANF temporal structure is compared in normotensive and hypertensive rat.

MATERIALS AND METHODS

72 normotensive and 36 hypertensive (SHR) male Sprague Dawley rats weighing 200-220g are placed single in special metabolism cages with separate urine and feces collection All experiments are located in a chronobiologic sound-attenuated box with controlled environmental conditions: constant temperature ($24-25°C$), atmospheric pressure (750-770 mmHg), hygrometry and light automatically controlled in light period 08.00-20.00, dark period 20.00-08 00. At six consecutive times (08.00, 14 00, 20.00, 02.00, 08 00 and 14 00) subgroups of six rats each are killed by decapitation and blood sample collected Urines are collected during 5 consecutive 6 hours periods (08 00-14 00, 14 00-20 00, 20.00-02.00, 02.00-08.00 and 08.00-14.00). Diuresis directly is measured, urinary and plasma electrolytes assessed by spectrophotometry and hormones dosed by radioimmunoassay. ANOVA analysis has been done from experimental chronograms and Cosinor analysis done to detect circadian rhythm and to quantify chronobiological variables for each parameter Then, values have been compared between normotensive and

RESULTS

In normotensive rats, results showed a great homogeneity Plasma renin activity, angiotensin II, converting enzyme, aldosterone, corticosterone, exhibited a circadian rhythm (period 24±2h) with peak values for all hormones at the beginning of the dark period (acrophases between 20.00 and 22.00). Plasma ANF concentration minimum is also observed during the first hours of the light period (fig 1) We found a potassium excretion peak in the early dark period hours as well as excreted aldosterone peak. Sodium excretion peak occured at the end of the dark period

In spontaneously hypertensive rats, all these components present similar variations In a macroscopic approach plasma aldosterone, renin activity, corticosterone rhythms remained unchanged; their peak levels occured at the beginning of the dark period. However mean plasma aldosterone and corticosterone concentrations were more elevated than in normotensive rats. But, ANF and angiotensin II rhythms seem to be affected. Mean angiotensin II concentrations have been increased and no circadian rhythm could be detected.ANF did not seem to display a circadian variation and becames infradian rhythm with a calculated period of about 16 hours and a greater variation amplitude
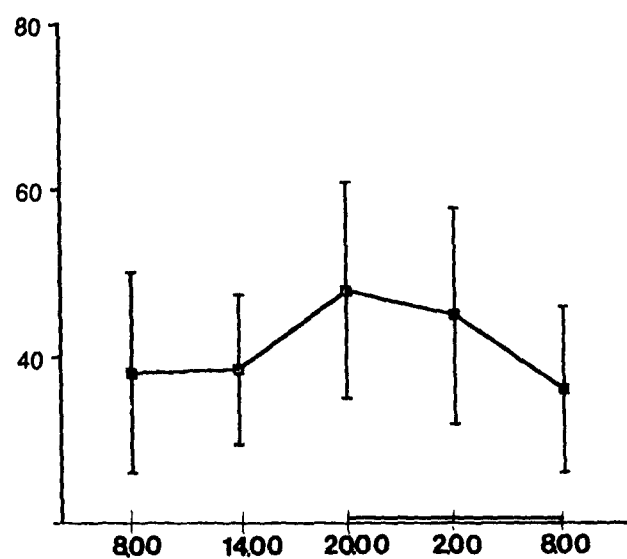
Fig. 1. Plasma atrial natriuretic factor circadian rhythm in normotensive
rat (NT) (in Fmol/ml)
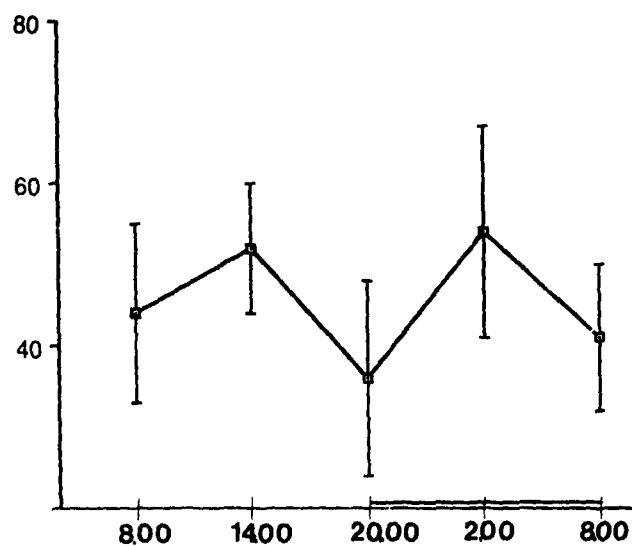
Fig. 2. Plasma atrial natriuretic factor circadian rhythm in hypertensive
rat (SHR) (in Fmol/ml).

So, ANF presented two peaks in the middle of the light (14 00) and of the dark period (02.00) (Fig.2) Urinary aldosterone excretion showed a circadian variation; but, the peak level has been shifted to the middle of the dark period.

DISCUSSION

Cosinor analysis permits to detect a circadian rhythm of the main RAS components, as well in normotensive rats as in SHR. In normotensive rat, all hormones present significant similar circadian changes with an acrophase at 20 00 for all of them , as it has been already reported (Hilfenhaus, 1976 - Laval, 1988 - Rodriguez-Sargent, 1982) Similar studies have been also reported for plasma renin and aldosterone in two kidney hypertensive rats (De Forrest, 1979). As it has been described for nocturnally-active species, the rhythm peaks occurs with a shift of about 12 hours compared to that seen in man. It is of interest to note that peak of ANF rhythm seems to be little shifted; indeed, in man, the peak value occurs at about 02 00 (Donckier et al., 1986). In SHR, all hormones present a similar circadian rhythm with a same acrophase except for ANF and angiotensin II Both hormones seem to be desynchronized, as very recent paper have reported also in patients with congestive heart failure (Yoshino, 1989) and hypertensive pregnant women (Sumioki, 1989) Moreover, we found no more circadian rhythm for plasmatic potassium and diuresis The regulation for electrolytes and water seem to be desorganized.

In conclusion, it could be carefully advanced that, in rat, ANF and angiotensin II could play the main role in genesis and in maintain of hypertension; ANF circadian rhythm change could be a fair marker to evidence hypertension. Such study concerning circadian variations of the different RAS components will permit so to better understand the structure and the physiology of the circulating and recently discovered local tissue renin angiotensin system (Dzau, 1987) Similar investigations should be fruitful in clinics to predict hypertension installation in man.

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THE METHOD OF MAXIMUM ENTROPIE (MME) SPECTRAL ANALYSIS IN THE EXAMINATION OF RHYTHMIC EFFECTS BY PLATELET ACTIVATING FACTOR (PAF) ON ARTERIAL BLOOD PRESSURE IN ANAESTHETIZED RATS

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All data treatments have to be consistent with respect to whole raw data as well as being objective as is possible with respect to unavailable data. This principle is not applied with "classical" spectral analysis methods. In fact, all unavailable data is zeroed (Theorem of Wiener-Kintchine) in which the autocorrelation function is biased and the associated spectrum erroneous. These serious limitations led us to use MME spectral analysis in the investigation of possible circadian rhythm effects of PAF on diastolic arterial blood pressure (PAD). PAF is a potent autocoid mediator implicated in a diverse range of human pathologies including asthma, shock, cardiac and systemic anaphylaxis, ulceration, transplant rejection, immune and renal disorders, hypotension, and a variety of inflammatory conditions.

PAD was measured every minute during 5 min. after an i.v.injection of PAF (125 and 50 ng/kg) and 30 sec. after an i.v. PAF dose of 5 ng/kg. At each time point, the mean PAD value of eight measurements on anaesthetized rats was determined. Experiments where performed at 2, 6, 10, 14, 18 and 22 h. during one day and for 4 different times during the year.

For example, the results obtained in june with a PAF dose of 125 ng/kg are:

Time	0 min	1 min	2 min	3 min	4 min	5 min
ME	74.5+1.68	41.50+0.84	49.54+1.23	61.12+1.62	70.46+1.66	76.46+1.58
AM	7.54+2.38	1.38+1.18	3.00+1.74	5.78+2.29	8.25+2.34	8.71+2.23
AC	-5.45+1.20	-2.20+3.29	-5.35+2.21	-5.62+1.51	-5.46+1.08	-5.46+0.98

ME = Mesor ; AM = Amplitude ; AC = Acrophase ;

As shown in the table, a daily rhythm is detected for normal arterial pressure which is not modified by PAF-induced hypotension. Similar results were found for the other dates and doses.

Taking into consideration circannual aspects, we remark that the acrophase of the observed daily rhythm occured between 2 and 6 a.m., dependent on the season. There is a significant maximum mesor in december, with regard to the three other months (march, june, september). It should be noted that the PAF time of recovery to a normal pressure after hypotension doesn't vary appreciably with season.

CHRONOPHARMACOKINETICS AND HEMODYNAMIC EFFECTS OF ORAL NIFEDIPINE IN HEALTHY SUBJECTS AND IN HYPERTENSIVE PATIENTS

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ABSTRACT

The daily variations in pharmacokinetics and hemodynamic effects on blood pressure [BP] and heart rate [HR] of oral nifedipine (NIF) were studied in healthy subjects and in hypertensive patients. NIF was applied either as an immediate-release preparation (10 mg) to 12 male healthy volunteers at either 08h or at 19h or as a sustained-release formulation (20 mg) to 5 hypertensive patients twice daily at 08h and 19h for 7 days. Significant daily variations were found in the pharmacokinetics of i.r. nifedipine after morning and evening dosing [Cmax: 82.0 ± 22.6 vs 45.7 ± 22.1 ng/ml; tmax: 22.5 ± 10.1 vs 37.5 ± 13.6 min]. Bioavailability of nifedipine (AUC) was reduced by about 35% after evening dosing. Ratio in AUC of metabolite to NIF was not circadian time dependent. Duration in BP decrease was more pronounced after morning dosing, that in HR increase after evening dosing. In contrast, no significant daily variations were found in the pharmacokinetics of s.r. NIF in hypertensive patients. 24-Hour values in BP were reduced by NIF, though peak effects in BP decrease and HR increase were not circadian time dependent.

KEY WORDS

Nifedipine i.r./s.r. - pharmacokinetics - hemodynamics - healthy and hypertensives - daily variations

INTRODUCTION

Various cardiovascular active drugs have been shown to display significant daily variations in their hemodynamic effects and/or in their pharmacokinetics (Lemmer, 1989). The aim of this study was to find out whether there was a circadian phase dependency in the pharmacokinetics of the calcium channel blocker nifedipine [NIF], too. Simultaneously, hemodynamic effects of nifedipine on systolic and diastolic blood pressure [BP] and on heart rate [HR] were registered by an automatic 24-h-monitoring device. Studies were performed with an immediate-release formulation of NIF in healthy subjects and with a sustained-release formulation in hypertensive patients.

MATERIAL AND METHODS

All subjects had an activity/rest cycle of 16:8 h, with nocturnal rest from 23.00 - 07.00h. In both studies subjects remained recumbent from 07.45-09.30h and from 18.45-20.30h.

In the first study a single dose of 10 mg of an immediate-release capsule of nifedipine (Cordicant[R] Kapsel, Mundipharma) was administered (bitten and swallowed) together with 100 ml of water to 12 healthy male volunteers (20-31 years) after 6 hours of fasting. Standardized meals were served 1.5, 5 and 12.5 hours after drug application. The drug was taken at 08.00 h or at 19.00 h local time with at least three days washout between the studies. Blood samples were taken before and 15, 30, 45, 60, 90, 120, 210, 300, 720, 960 and 1380 minutes after drug intake (Lemmer et al, 1989).

In the second study 5 hypertensive patients until now received a sustained-release formulation of NIF (Cordicant[R] retard, 20 mg) twice daily at 08.00h and at 19.00h for 7 days and blood samples were taken on the 7th day over 24 hours. Standardized meals were served at 09.30h, 13.00h and at 20.30h.

Serum concentrations of NIF and its main nitropyridine metabolite were determined by gaschromatography with ECD detection.

In both studies systolic and diastolic blood pressure and heart rate were frequently registered by an automatic, portable device (Physioport[R]). For each healthy subject or hypertensive patient, circadian control values in blood pressure and heart rate were registered on a separate occasion either within one week before first drug study (healthy subjects) or after a one week washout period before subacute treatment with NIF (hypertensive pts).

The following pharmacokinetic parameters were calculated: Cmax, tmax, AUC and $t_{1/2\beta}$. Peak drug effects on BP decrease and HR increase [Emax] and time to peak drug effects [Tmax] were determined in relation to the circadian controls. In addition, the cumulative drug effect within the first three hours (healthy subjects) or from 08.00-19.00h and from 19.00-06.00h (hypertensive pts.) was determined by the area under the effect-time-curve (AUC_{0-th}) and compared with respective circadian control values.

Significance of daily variation in pharmacokinetic parameters was tested by student's t-test for paired data or Mann-Whitney-U-test. Significance of daily variations in hemodynamic effects was tested by one-way ANOVA and rhythmicity by cosinor method (Witte et al, 1989). The limit of significance was $p < 0.05$. Both studies were approved by the Ethical Committee, Medical Faculty of the J.W.Goethe-University.

RESULTS

Significant daily variations were found in the pharmacokinetics of the i.r. formulation of NIF after acute dosing (10 mg) to healthy subjects (Tab.1).

	Time of Nifedipine Application		
	08.00h	19.00h	
Cmax [ng/ml]	82.0 ± 6.5	45.7 ± 6.4	•••
tmax [min]	22 5 ± 2.9	37.5 ± 3.9	••
AUC_{0-than} [ng/ml•h]	130 ± 13	85 ± 13	•
Cmax/tmax [ng/ml/h]	4.5 ± 0.7	1.5 ± 0.3	•••
t½ ß [min]	64.6 ± 8.8	57.7 ± 6.0	n.s.
AUC_{0-than} metabolite/AUC_{0-than} nifedipine:			
	0.49 ± 0.16	0.52 ± 0.08	n.s.

• $p < 0.05$, •• $p < 0.01$, ••• $p < 0.001$

TAB.1. Pharmacokinetics of i.r. nifedipine (10 mg) after oral dosing at 08.00h or at 19.00h to 12 healthy volunteers. Mean ± SEM.
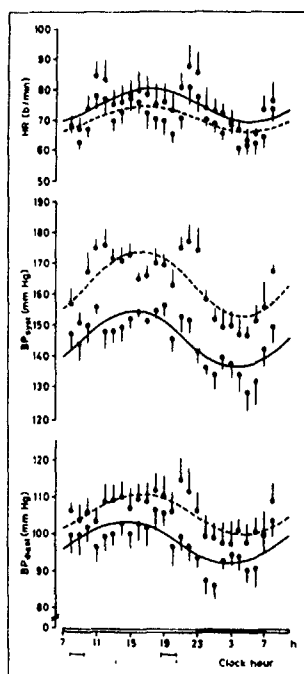
Fig.1. Cosine fit of hourly values in blood pressure and heart rate before (thick line) and on the 7th day of treatment (broken line) with 20 mg s.r. NIF twice daily at 08.00h and at 19.00h, mean values ± SEM.

| | Time of s r. Nifedipine Application on 7th Day | | Day |
	08 00h	19 00h	p
Nifedipine.			
Cmax [ng/ml]	46.5 ± 12.3	58.4 ± 12 3	
tmax [h]	2.3 ± 0 4	2 9 ± 0.4	>0.05
AUC$_{0-11h}$ [ng/ml·h]	304 8 ± 74.6	365.0 ± 92.5	
Nitropyridine Metabolite·			
Cmax [ng/ml]	19.4 ± 4.1	22.0 ± 5.3	
tmax [h]	1 9 ± 0 4	2.1 ± 0 6	
AUC$_{0-11h}$ [ng/ml·h]	106.4 ± 19 4	123.7 ± 23.4	

TAB.2. Pharmacokinetics of s.r. nifedipine after 7 days treatment with 20 mg twice daily (08.00h, 19.00h) in 5 hypertensive patients. Mean ± SEM.

Even bioavailability of the i.r. NIF was reduced by about 35% in the evening as measured by the AUC (Tab.1). However, the ratio in AUC of the nitropyridine metabolite to the parent compound was not significantly different at the two time points, indicating no circadian phase dependency in drug metabolism. Whereas peak drug effects [Emax] on BP and HR and time-to-peak effects [Tmax] were not circadian phase dependent, duration in BP decrease within first 3 hours was

significant only after morning application and duration in HR increase was greater after evening dosing.

In the hypertensive patients so far investigated the retard formulation of NIF did not display daily variations in its pharmacokinetics (Tab.2). In all of the patients blood pressure was reduced by NIF treatment as indicated by a decrease in mesor in systolic and diastolic BP. Mesor in HR was increased in 3, decreased in one and not changed in one patient (see Fig.1).

Peak drug effects [Emax] were again not circadian phase-dependent (Tab.3), time to peak effects [Tmax] may be slightly shorter after evening than after morning dosing (Tab.3).

	Time of s.r. Nifedipine Application on 7th Day		
	08.00h	19.00h	p
Blood Pressure syst			
Emax [mm Hg]	-64.2 ± 11.2	-58.2 ± 12.3	
Tmax [h]	2.9 ± 0.4	2.2 ± 0.5	
Blood Pressure diast			
Emax [mm Hg]	-27.2 ± 5.2	-38.2 ± 5.3	
Tmax [h]	3.1 ± 0.3	1.6 ± 0.2	<0.05
Heart Rate			
Emax [b/min]	22.8 ± 5.6	21.2 ± 4.1	
Tmax [h]	2.2 ± 0.5	1.9 ± 0.2	

TAB.3. Peak effects [Emax] and time to peak effects [Tmax] on blood pressure and heart rate of 5 hypertensive patients after 7 days of treatment with s.r. nifedipine (2x20 mg).

DISCUSSION

The data obtained in the healthy volunteers clearly demonstrate a pronounced circadian phase dependency in the pharmacokinetics and in the hemodynamic effects of oral immediate-release nifedipine. Most important, also bioavailability was significantly reduced after evening application. It is assumed that this is due to presystemic drug degradation, because the relative amount of metabolite formed was not different at the two time points of drug application. In contrast, the sustained-release formulation of nifedipine did neither display daily variation in pharmacokinetics nor in hemodynamic effects in hypertensive patients after subacute drug treatment. These data could indicate that the galenic formulation is also of importance whether or not a drug exhibits a chronokinetic behaviour. This assumption is supported by similar chronopharmacokinetic findings with an immediate-release and a sustained-release formulation of isosorbide-5-mononitrate (Lemmer et al, 1989).

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CHRONOBIOLOGIC DIFFERENCE IN THE EFFICACY OF ANTIHYPERTENSIVE AGENTS TO ESSENTIAL "HYPERTENSION"

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ABSTRACTS

1) The antihypertensive effects of nicardipine hydrochloride, metoprolol and captopril were validated comparing with the time qualified reference interval from our data base.
2) The mode of the efficacy of the agents on the circadian characteristics of blood pressure was clearly different each and all.
Therefore, these difference should be considered for the selection of the antihypertensive agents for the individualization of the long term therapy of "hypertension".

KEYWORDS

Nicardipine hydrochloride; metoprolol; captopril; circadian rhythm of blood pressure.

INTRODUCTION

The development of the hardware for the long term ambulatory blc d pressure monitoring enabled us to evaluate the time sequential changes of blood pressure. Since the changes of blood pressure can be monitored in each individual, the individualization of the diagnosis and treatment for hypertension has been assessed. However, with the traditional concept and approach of the medicine these can not be solved. The chronobiologic concept, software and data base can break through these barriers.
Therefore, the aim of this study was to evaluate the chronobiologic efficacy of the antihypertensive agents, such as nicardipine hydrochloride (calcium antagonist), metoprolol (adrenergic $\beta 1$ receptor antagonist), and captopril (angiotensin converting enzyme inhibitor). Are they all the same regarding the effect on blood pressure changes including the circadian rhythm characteristics of blood pressure and heart rate to control essential hypertension?

METHODS AND SUBJECTS

Blood pressure and heart rate were monitored with an ambulatory instrument (ABPM 630 manufactured by Nippon Colin Ltd., Japan) every 30 minutes for 48 hours under ordinary conditions of life. Patients were asked to stay in bed from 23:00 to 07:00 and to take standard meals at regular times during monitoring. The data were transferred to Nippon Electric Co. computer system and analyzed convention-

No 55 T.T, 52 y o, female

(1) Before treatment

(2) After Nicardipine hydrochloride, 20mg, t.i.d. for 2wks.

HBI = hyperbaric index
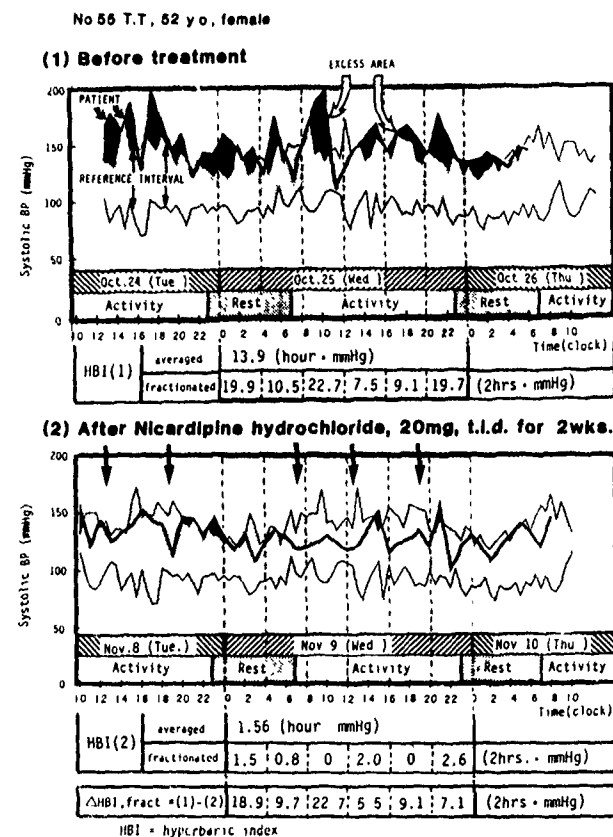
Fig. 1. A representative case of the evaluation of the rhythm pattern in essential hypertension. The magnitude of the antihypertensive effect was examined as the averaged and fractionated HBI. These indices were examined before the therapy and after the administration of nicardipine hydrochloride (20mg of three times-daily) for two weeks.

ally and chronobiologically. The conventional BP data analysis for systolic, diastolic pressure and heart rate were performed. The least-squares-fit of a cosine curve yielded estimates of the circadian midline estimating statistic of rhythm (MESOR), amplitude and acrophase for each variables. The data were evaluated by reference rhythm (time qualified reference interval) derived from our 334 clinically healthy "normotensive" Japanese volunteers, using proper homemade software (1,2) (Fig. 1.). The protocol of this study was as follows. The first monitoring was performed for 48 hours before any treatment. All together 31 cases of "essential hypertension" based on "WHO criteria" of "hypertension" were evaluated. In nicardipine hydrochloride (N) 13 patients were subsequently started on a twice-daily regimen of long acting formulation of nicardipine for one week. Thereafter, a second monitoring of blood pressure for 48 hours was performed. In captopril (C), 12.5mg of three times-daily regimen was continued for 3 weeks and then the dosage was increased to 25mg of three times -daily for 4 weeks. 9 cases were examined. In metoprolol (M), 40mg of three times was given daily for 4 weeks to 9 cases. Each drug was given to the different patient at different times.

RESULTS

1) The conventional statistical analysis:
The actually measured values were compared with the reference rhythms as shown in the Fig. 1. In this figure, the magnitude of the deviant pressure are indicated as the area in black. This area in excess was integrated and expressed as averaged time area in one hour. Furthermore, the time fractionated hyperbaric index in every four hours were also calculated to examine the efficacy of the chronotherapy. In this representative case, N was valid as the chronotherapy since the efficacy can be easily recognized from the numbers in the figure. A) Mean values: In all tested cases, 48 hours mean values were lower in all drugs significantly (P<0.01). The mean values of systolic and diastolic pressures both in sleep and awake spans decreased significantly. Similarly heart rate decreased

Fig. 2. The differences of the effects of antihypertensive agents on midline estimating statistic of rhythm of blood pressure and heart rate.
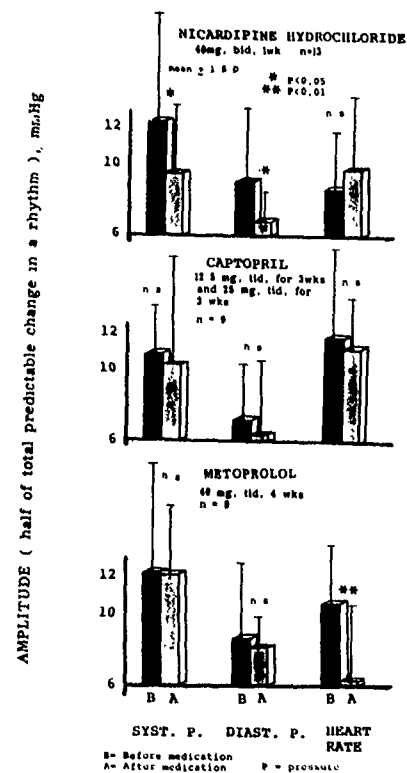
Fig. 3. The differences of the effects of antihypertensive agents on circadian rhythm amplitude of blood pressure and heart rate.

except mean values of N in awake span. B) Baric indices; The hyperbaric index of systolic pressure decreased significantly in all agents. This index of diastolic pressure decreased also in N and M even though C did not lowered pressure. The heart rate remained same. The hypobaric indices in systolic and diastolic pressures remained same in all agents. However, the hypobaric index of heart rate in M decreased even though the rest of the agents did not show these decreases.

2) The cosinor analysis.
The cosinor data of each and all variables are shown not only as the calculated curves. According to these chronobiologic analysis, MESOR of systolic and diastolic pressures (Fig. 2.) decreased in all agents significantly. However, MESOR of heart rate by N remained same. But those of C and M decreased significantly. Furthermore, the circadian amplitudes of rhythm (Fig. 3.) of systolic and diastolic pressure were lowered by N, even though this decrease was not in C and M. The amplitude of heart rate decreased by M, even though N and C did not affect the amplitude. The acrophase did not change at all in any of the agents tested.

DISCUSSION

The effect of antihypertensive therapy on the circadian blood pressure pattern

has been evaluated because whether treatment was effectively regulating blood
pressure the entire day (3). The efficacy of antihypertensive agents regarding
the mode of antihypertensive effect including the circadian rhythm character-
istics should be defined before the most effective method of blood pressure
reduction. Ho· r, there is no comparable studies regarding these effects· In
beta-blockers, the difference in mode of the action was evaluated collecting the
reports in the literature (4), even though the clinical and physiological
significance remained to be defined.
The efficacy of nicardipine, calcium antagonist, has been validated (5,6). In
order to perform the individualized therapy to hypertensive subjects as "chrono-
therapy", the chronobiologic evaluation of nicardipine was valid. In this drug
the monotherapy was performed employing a long-acting formulation with the
combination of a short acting formulation to enhance the desired pharmacologic
effect according to the magnitude of the outlying area of blood pressure to the
reference range. The mode of action of nicardipine was rather uniform compared
with the rest of the agents. However the monotherapy by this agent was
promising.
The efficacy of metoprolol was also confirmed. The original circadian character-
istic of blood pressure was well preserved compared especially with nicardipine
after the treatment. However, these characteristics of the heart rate were
changed after the treatment. Captopril showed the definite antihypertensive
effect (7) preserving the circadian characteristics not only of blood pressure
but also the heart rate. This was in good contrast comparing with nicardipine
and metoprolol.
The physiological and clinical significance on these findings regarding the mode
of the action including the circadian rhythm remains to be explored. However,
these circadian characteristics should be emphasized to obtain the full and
complete information on blood pressure changes to develop the rational indivi-
dualized chronotherapy for hypertensive subjects.

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CHONOBIOLOGIC EVALUATION OF THE EFFICACY OF CAPTOPRIL IN ESSENTIAL "HYPERTENSION"

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ABSTRACT

The efficacy of captopril in 9 patients with essential "hypertension" was validated with conventional statistical method and cosinor method. Captopril showed the definite antihypertensive effect reserving the circadian rhythm of blood pressure (BP) and heart rate (HR).

KEYWORDS

Captopril; essential hypertension; cosinor method; circadian rhythm.

INTRODUCTION

The efficacy of captopril, an angiotensin-converting enzyme (ACE) inhibitor, has been validated in hypertension (1). The characteristic circadian rhythm exists in BP (2,3). Therefore, the chronobiologic evaluation has been thought to be necessary for the assessment of the efficacy of antihypertensive agents. Therefore, the purpose of this study was to assess the efficacy of captopril chronobiologically in the treatment of essential hypertension.

SUBJECTS AND METHODS

The subjects of this study were the outpatients with essential hypertension based on WHO criteria. Namely, BP of more than 140/90 mmHg was diagnosed to be hypertensive. Their age ranged from 42 to 78 years (mean age 60). Three were male and 6 were female. The ambulatory BP was monitored with instrument of ABPM-630 of Nippon Colin in Japan. The first monitoring was performed every 30 minutes for 48 hours in their daily activities before the treatment. Captopril was administered at least for 6 weeks continuously. The starting dose was 12.5mg, three times-daily. If BP was not lowered enough after 3 weeks of medication, in the next 3 weeks the dose was increased to 25mg, three times-daily. Thereafter, the second monitoring was performed quite similarly to the first.
These data were analyzed with A) conventional statistical method. The mean values of systolic, diastolic BP and HR were calculated. These data were evaluated with chronodesm obtained from healthy normotensive Japanese volunteers of 334 subjects (4). In order to assess the efficacy of captopril quantitatively, the hyper-/hypo-baric indices of BP and HR were calculated (5,6). Furthermore, B) chronobiologic analysis (cosinor method) was performed. The least square fit of a cosine curve with a period of 24 hours was calculated to obtain, MESOR
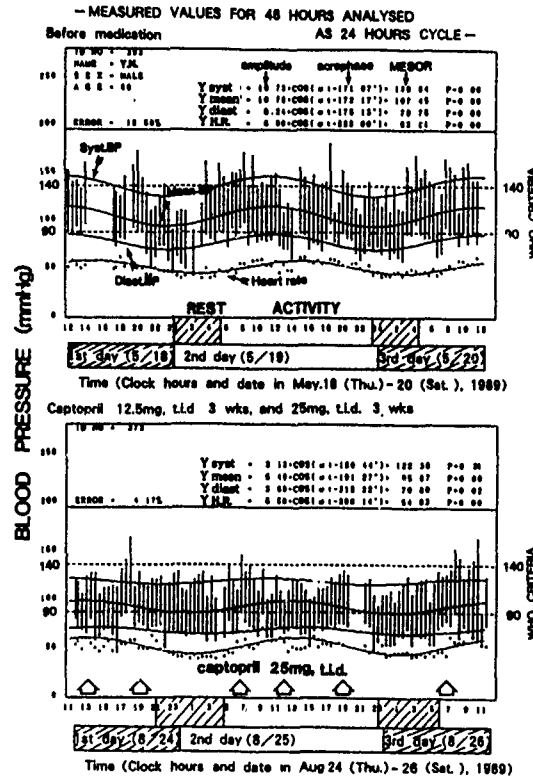
Fig. 1. A representative case treated with captopril.

(midline estimating statistic of rhythm), amplitude and acrophase.

RESULTS

A) Conventional statistical method:
a) Mean values.
A representative case is shown in Fig. 1. The upper panel shows the changes of BP and HR before the treatment. The lower panel shows those changes after the treatment. The changes of systolic, mean and diastolic BP were indicated with bars. The changes of HR were shown with black points. Before the treatment, this patient clearly snowed hypertension based on the conventional data compared with WHO criteria in awake span. Captopril was administered to this patient for 6 weeks. Initial dose was 12.5mg, (tid.) for the first 3 weeks, and in the next 3 weeks the dose was increased to 25mg, (tid.). The effect of captopril was definitely noted. Both systolic and diastolic BP were lowered less than WHO criteria.
b) Baric indices.
In order to assess the efficacy of captopril the averaged hyper- and hypo-baric indices were calculated. The 90% time specified reference limits derived from our data base is shown as the gray band in Fig. 2. When the black outlying area is compared with this chronodesm, it became to recognize easily both visually and quantitatively. Before the medication, this patient clearly showed hypertension. After the treatment, most of the black areas of BP disappeared, and fortunately the averaged deficit area did not change. A summary of the efficacy of captopril is shown in Table 1. Systolic and diastolic BP definitely decreased after the treatment in the mean values during 48 hours, the sleep span and the awake span. HR decreased slightly after the treatment. The hyperbaric index of systolic BP
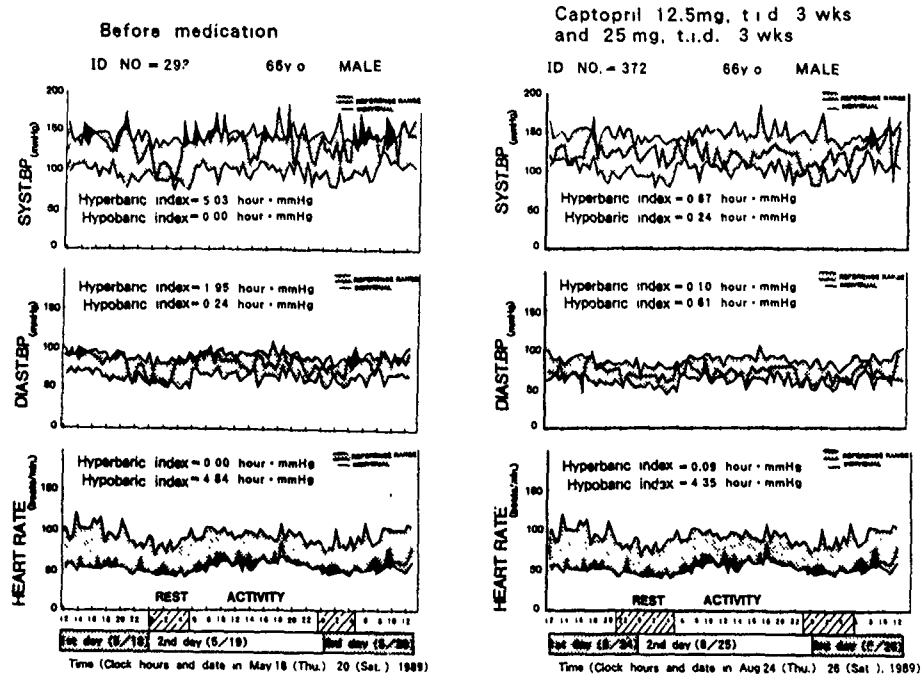
Fig. 2. Efficacy of captopril with averaged
hyper-/hypo-baric indices

Table 1 Summary of the efficacy of captopril in
essential hypertension

	SYSTOLIC BP		DIASTOLIC BP		N = 9 HEART RATE	
	before	after	before	after	before	after
Mean of 48 hours	mmHg 136.3 ± 11.4	129.2 ± 8.6	mmHg 78.9 ± 8.8	72.6 ± 7.0	b.p.m. 73.5 ± 10.2	68.4 ± 6.6
Mean in sleep span	134.2 ± 14.3	121.7 ± 14.3	74.7 ± 8.7	67.3 ± 8.8	63.9 ± 9.6	57.9 ± 7.3
Mean in awake span	140.5 ± 11.2	132.8 ± 7.3	80.9 ± 8.7	75.9 ± 7.1	78.0 ± 10.6	72.7 ± 7.0
Hyperbaric index	hour · mmHg 6.97 ± 6.01	3.03 ± 2.77	hour · mmHg 2.77 ± 3.53	1.06 ± 1.22 N.S.	hour · mmHg 1.23 ± 1.69	0.34 ± 0.42 N.S.
Hypobaric index	0.24 ± 0.41 N.S.	0.34 ± 0.47	0.17 ± 0.18 N.S.	0.39 ± 0.46	0.55 ± 1.60 N.S.	0.51 ± 1.44
MESOR	mmHg 138.1 ± 11.1	129.0 ± 8.8	mmHg 78.8 ± 8.5	72.5 ± 7.0	b.p.m. 73.0 ± 9.5	68.6 ± 6.8
Amplitude	10.0 ± 2.7 N.S.	9.3 ± 6.0	6.2 ± 3.1 N.S.	5.4 ± 4.2	10.9 ± 4.4 N.S.	10.3 ± 2.9
Acrophase	degree - 170.8 ± 66.5 N.S.	- 200.6 ± 48.3	degree - 170.2 + 74.7 · S.	- 212.7 ± 44.0	degree - 218.8 ± 16.5 N.S.	- 219.5 ± 21.9

mean ± 1.S.D * P < 0.05 * * P < 0.01

decreased, but the hypobaric indices did not change.
B) Cosinor analysis:
The cosine curves of BP and HR of this case were shown in Fig. 1. In this way the circadian rhythms were recognized in the variation of BP and HR in all patients. MESOR of systolic, diastolic BP and HR decreased after the treatment. These values were quite similar to the mean values of 48 hours. On the other hand, amplitude and acrophase of BP and HR did not change.

DISCUSSION

The efficacy of captopril in essential hypertension were validated with conventional statistical method and chronobiological method (cosinor analysis). Captopril showed definite antihypertensive effect all day long. The hyper- and hypo-baric indices enable us to evaluate this effect visually and quantitatively. It was estimated that captopril may influence the circadian rhythm of BP, since the variation of BP is regulated by the autonomic nervous and the endocrine system. However, captopril did not change amplitude and acrophase of BP. Therefore, the circadian rhythm of BP was well preserved in spite of the antihypertensive effect. We have reported that metoprolol, an adrenergic 1 antagonist, also did not influence the circadian rhythm of BP (7). On the other hand, according to our evaluation of nicardipine hydrochloride, a calcium antagonist(8), amplitude of BP decreased and the circadian rhythm of BP changed. Therefore, it is noteworthy that there are differences between antihypertensive agents in the effect on the circadian rhythm of BP.

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CHRONOBIOLOGIC EVALUATION OF THE EFFICACY OF METOPROLOL TO ESSENTIAL "HYPERTENSION"

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ABSTRACT

The efficacy of metoprolol in essential "hypertension" was validated. The conventional statistical analysis of blood pressure and heart rate showed definite decrease of blood pressure. Furthermore, the cosinor analysis of those showed the original circadian characteristics of blood pressure was well preserved even after the treatment, however, the amplitude of heart rate decreased.

INTRODUCTION

The ambulatory long term monitorings of blood pressure and heart rate have been popular in the clinical practice. Everybody agree to perform this approach for the evaluation of "hypertension". However, there are certain limitation how to evaluate this time sequential changes of blood pressure.
In order to break through these barriers, it is necessary to accept the new concept of chronobiology and chrono-engineering. The clinical application of this new science and technology enabled us to approach to this problem (1,2). Primary prevention with metoprolol, adrenergic β1 antagonist, has been definitely proved (3,4). However, in order to individualize the diagnosis and treatment to "hypertension" the mode of the efficacy of metoprolol on the rhythm characteristics of blood pressure and heart rate is necessary (4).
Therefore, the object of this study was to evaluate the mode in action including rhythm characteristics of blood pressure and heart rate of β1 receptor antagonist, metoprolol, in essential hypertension.

METHODS AND SUBJECTS

Blood pressure and heart rate were monitored with an ambulatory instrument (ABPM 630 manufactured by Nippon Colin Ltd., Japan) every 30 minutes for 48 hours under ordinary condition of life. Patients were asked to stay in bed from 23:00 to 07:00 and to take standard meals at regular time during monitoring period. The data were transferred to Nippon Electric Company computer system. They were analyzed for systolic, diastolic pressure and heart rate as shown in Fig. 1. The least square fit of a cosine curve yielded estimates of the circadian midline estimating statistic of rhythm (MESOR), amplitude and acrophase for each varia-
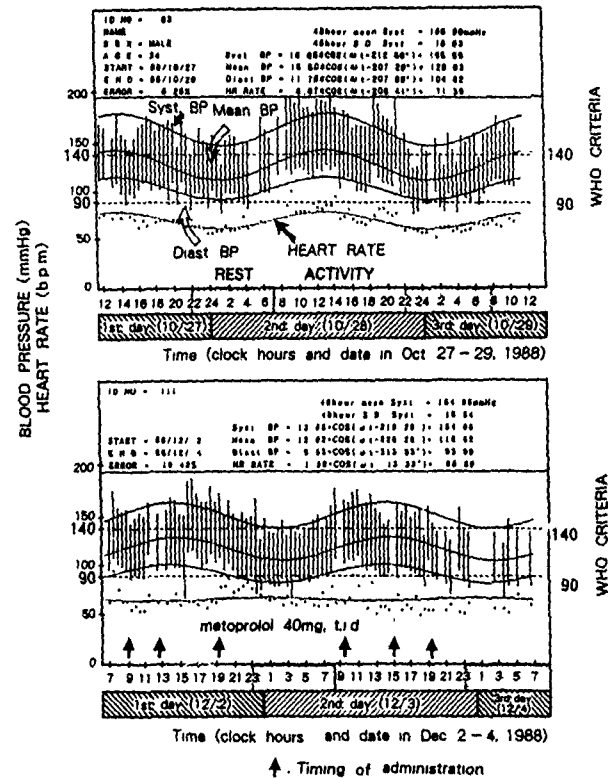
Fig. 1. Conventional and cosinor analysis of blood pressure and heart rate measured for 48 hours.

bles. The data were evaluated by reference rhythm (time qualified reference interval) derived from our 334 clinically healthy "normotensive" Japanese volunteers, using proper homemade software (1,2) (Fig. 1.). The protocol of this study was as follows. The first monitoring of this study was performed for 48 hours before any treatment. Metoprolol(M) of 40mg in three times-daily was given for 4 weeks continuously. Thereafter, the monitoring was repeated. 9 cases (6 male, 3 female, age of 31 to 71 years) of essential "hypertension" based on "WHO criteria" were evaluated.

RESULTS

1. Conventional statistical analysis
A) Mean values(Table 1).
In order to evaluate the mode of the action of metoprolol as a antihypertensive agent, blood pressure and heart rate were monitored for 48 hours (Fig. 2.). The actually measured values were compared with the reference rhythms. In order to evaluate the magnitude of the deviant pressure and heart rate, the averaged hyper-/hypo-baric indices were calculated based on our chronodesm (1). as shown in a representative case of Fig. 2., time averaged hyperbaric index of systolic pressure decreased from 27.1 to 14.55 mmHg x hour. Furthermore, diastolic pressure also decreased from 20.45 to 8.75 mmHg x hour index. Therefore metoprolol showed definite antihypertensive effect. However, heart rate did not change after the treatment. The mean values of 48 hours of systolic, diastolic pressure and heart rate decreased significantly after metoprolol. Furthermore, the mean values of those in awake and sleep spans decreased significantly in a similar fashion. There were no significant differences between the magnitudes of blood pressure between in awake and sleep spans. B) Baric indices (Table 1): The hyperbaric index in systolic and diastolic pressure decreased significantly. However, this index did not change at all in heart rate. The hypobaric index of systolic and diastolic pressure did not change after metoprolol. Therefore, metoprolol did not decrease pressures more than the reference rhythm. However, the hypobaric index of heart rate decreased significantly. Therefore, metoprolol effect on heart rate was remarkably different from the effect of blood pressure.

2) Cosinor analysis (Table 1).
MESOR of systolic, diastolic pressures and heart rate decreased significantly after metoprolol. The circadian amplitude of systolic and diastolic pressures did not change after the treatment compared with pre-treatment data. This amplitude, however, decreased significantly in heart rate. The acrophase of

Table 1   The summary of effects of metoprolol evaluated by
conventional statistic method and by cosinor method

	SYSTOLIC BP		DIASTOLIC BP		HEART RATE		N=9
	before	after	before	after	before	after	
I. 1. Mean of 48 hrs.	144±14	132±15 **	86±11	77±11 **	72±11	62±8 **	
2. Mean in awake span (mmHg)	150±15	138±16 **	91±11	81±11 **	77±10	65±8 **	
3. Mean in sleep span (mmHg)	132±16	121±15 *	77±12	69±10 *	62±13	55±10 *	
4. Hyperbaric index (mmHg hour)	9.5±9.1	5.8±5.7 *	6.0±6.7	3.0±4.0 *	0.9±1.9	0.3±0.3 N.S.	
5. Hypobaric index (mmHg hour)	0.1±0.1	0.2±0.2 N.S.	0.1±0.1	0.4±0.7 N.S.	0.3±0.6	1.6±1.8 *	
II. 1. MESOR (mmHg)	144±14	132±15 **	86±11	77±11 **	72±11	62±8 **	
2. Amplitude (mmHg)	12.2±6.1	12.1±3.8 N.S.	8.6±4.1	8.1±1.7 N.S.	10.5±3.2	6.3±4.1 **	
3. Acrophase (°)	225±35	207±37 N.S.	227±37	197±48 N.S.	213±15	194±73 N.S.	

MESOR:midline-estimating statistic of rhythm   *:P<0.05
                                                  **:P<0.01

BEFORE MEDICATION   AFTER METOPROLOL
(40 mg, TID, 4 WEEKS)

SYSTOLIC BLOOD PRESSURE

DIASTOLIC BLOOD PRESSURE

HEART RATE

TIME (clock)   TIME (clock)
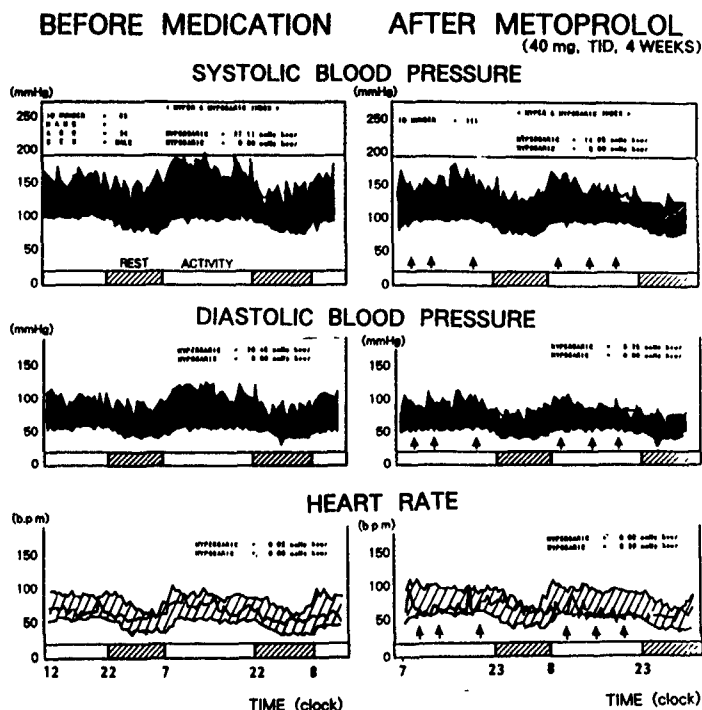
Fig. 2.   A representative case showing the efficacy of metoprolol

systolic, diastolic and heart rate did not change at all even after the treatment.
In order to assess the magnitude of outlying pressure compared with rhythm reference of blood pressure, the baric indices were evaluated in metoprolol. The hyperbaric index of systolic and diastolic pressures decreased significantly. However, this index of heart rate did not change. These changes were different from nicardipine hydrochloride and captopril (2,9). The hypobaric index of systolic and diastolic pressures didn't change. However, this index of heart rate decreased significantly. In order to examine the rhythmic change as a whole and to know the trend of blood pressure changes, the cosinor analysis of circadian

rhythm of blood pressure was performed. MESOR didn't change after metoprolol.
These values were similar to the mean values of 48 hours.
The amplitude and acrophase of systolic and diastolic pressures didn't change.
Therefore, the blood pressure rhythm after metoprolol was well preserved in spite
of the effect of the lowering blood pressure as a whole. However, MESOR and
amplitude of heart rate changed after metoprolol. This heart rate rhythm was
remarkably changed after this treatment. The acrophase of metoprolol did not
change as similarly noted in nicardipine hydrochloride and captopril.
Even though the physiological and clinical significance on these finding
regarding the mode of rhythm remained to be examined, these characteristics
should be emphasized to establish the individualization of antihypertensive
therapy.

DISCUSSION

It has been accepted that the ambulatory blood pressure monitoring became a
standard method of clinical trial of antihypertensive efficacy. The chrono-
diagnosis of "hypertension" became feasible assessing the changes of the biologic
time structure due to deviant blood pressure. With the combination of these two
recent achievements in chronobiology the administration of antihypertensive
agents was individualized considering the treatment time of sensitivity
resistance cycles of hypertension to enhance the pharmacologic effects of
antihypertensive agents. The software for the analysis of blood pressure to
quantitative the rhythmic changes is not universally accepted yet. In order to
perform the conventional statistical analysis several proposal have been made.
24 hours mean value of blood pressure is a index examined in this study. The
decrease of 24 hours mean value was recognized in metoprolol.
Since the rhythm of metoprolol is mediated by the sympathetic nervous system, the
antihypertensive effect was examined separating awake and sleep spans. The
effects were similarly effective in both spans.

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CIRCADIAN VARIATION OF NEW CARDIAC HORMONES THAT LOWER BLOOD PRESSURE AND INCREASE SODIUM EXCRETION

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ABSTRACT

The N-terminus of the atrial natriuretic prohormone (ANF) prohormone contains two peptides consisting of amino acids (a.a.) 1-30 and 31-67 while the C-terminus contains 1 peptide (a.a. 99-126) of this 126 a.a. prohormone which lower blood pressure and have natriuretic properties. To determine if either the N-terminus and/or the C-terminus of the ANF prohormone have a circadian variation in their circulating plasma concentrations 3 men, ages 41-47, who have been followed for 19 years with respect to circadian variation in physiological variables including blood pressure and clinical chemistries had blood sample obtained for radioimmunoassay every 3 hours during a 24 hour period. Three radioimmunoassays which immunologically recognize 1) the whole N-terminus (i.e. a.a. 1-98), 2) the midportion of the N-terminus (a.a. 31-67) and 3) the C-terminus (a.a. 99-126) of the ANF prohormone were utilized. The whole N-terminus, the midportion of the N-terminus which circulates after being proteolytically cleaved from the rest of the N-terminus, and the C-terminus each had a peak circulating concentration between 0400 and 0700 which were significantly ($P<0.001$) higher than their concentrations at any other time throughout the 24 hour period. It was concluded that there is a circadian rhythm in both the N-terminus and C-terminus of the ANF prohormone with peak plasma concentrations near the 24 hour nadir in blood pressure suggesting a possible cause for this blood pressure nadir since both the N-terminus and C-terminus of this prohormone contain blood pressure lowering peptides.

KEYWORDS: Atrial natriuretic factor, blood pressure, N-terminal prohormone peptides.

INTRODUCTION

Recently several peptides from the 126 amino acid (a.a.) prohormone of atrial natriuretic factor (ANF) have been found to have potent natriuretic, diuretic, and blood pressure lowering properties (Martin et al., 1990). These peptides consisting of a.a. 1-30 (pro ANF 1-30; Long acting sodium stimulator), a.a. 31-67 (pro ANF

31-67; Vessel Dilator) and a.a. 99-126 (ANF; C-terminus of prohormone) all circulate normally in men with pro ANF 1-30 circulating as part of the 98 a.a. N-terminus while pro ANF 31-67, the midportion of N-terminus, and ANF circulate as separate entities (Winters et. al., 1989). The present investigation was designed to determine if there is a circadian variation in the circulating concentrations of the whole N-terminus (pro ANF 1-98), the midportion of the N-terminus (pro ANF 31-67), and the C-terminus (ANF) of the atrial natriuretic factor prohormone in 8 men, 41-47 years of age, who have been followed for 19 years with respect to circadian variation in blood pressure and clinical chemistries.

METHODS

Radioimmunoassays for pro 1-98, pro ANF 31-67 and ANF:

Radioimmunoassays to measure the N-terminus of the prohormone were devised to amino acids 1-30 and 31-67 of the 126 amino acid prohormone while the C-terminal assay measures amino acids 99-126 of the prohormone, i.e. ANF, as previously described by our laboratory (Winters et. al., 1989). Our pro ANF 1-30 immunologically recognizes the whole 98 a.a. N-terminus while pro ANF 31-67 measures a.a. 31-67 (Winters et. al., 1989).

RESULTS

Each subject showed a large range of change (ROC) from lowest to highest value in each pro ANF segment over the 24-hr observation span. The group average ROC for pro ANF 1-98 was 107%, for pro ANF 31-67 it was 115% and for the C-terminus (ANF; 99-126) it was 76%. The mean serum concentrations of pro ANF 1-98 measured every 3 hours during a 24-hr span are illustrated in Fig.1. As observed in this fig. there was a marked increase in the whole N-terminus at 0400. This elevation of the N-terminus remained elevated at 0700 and then decreased by one-half at 1000. Pro ANF 31-67 circulating concentrations followed a pattern almost identical to pro ANF 1-98. (Data not shown). The circulating concentrations of pro ANFs 1-98 and 31-67 at 0400 and 0700 were significantly increased (P<0.001) compared to all other points throughout the 24 hour period.

Atrial natriuretic factor followed a similar pattern. (Data not shown). In these eight healthy volunteers ANF was nearly double at 0400 its concentration at 2200. ANF was still markedly increased at 0700 and then decreased by 1000 to its circulating concentration observed at 2200. The high-amplitude circadian rhythms of both the 'N- and C-terminus of ANF prohormone were highly significant by both analysis of variance and cosinor techniques as observed in Fig.1.

DISCUSSION

The present investigation demonstrates that the whole N-terminus, the midportion of the N-terminus (i.e. pro ANF 31-67), and the C-terminus (i.e. ANF) of the ANF prohormone each have similar circadian rhythms with an approximate doubling of these peptides at 0400 compared to 2200. Atrial natriuretic factor's rhythm, as pointed out by (Halberg et al.,.1986), is similar to cortisol in its anticipatory circadian periodic rise prior to awakening, preparing for each day's activity. The same relationship to cortisol is true for the N-terminus of the ANF prohormone as observed in the present investigation.

Circadian Rhythm in
Serum Prohormone ANF 1-98
for 8 Clinically-Healthy Men in their 40's

For Time Effect
ANOVA: F = 240.4, p = <0.001
Mean Cosinor p = <0 001
MESOR 2149 ± 68
Amplitude 34% (27, 41)
Acrophase. 0600 (0540, 0620)

Prohormone Atrial Natriuretic Peptide 1-98 (pg/ml); Mean ± SE

3500
3000
2500
2000
1500

Activity | Rest | : Activity

19   22   01   04   07   10   13   16

TIME (local clock hour)
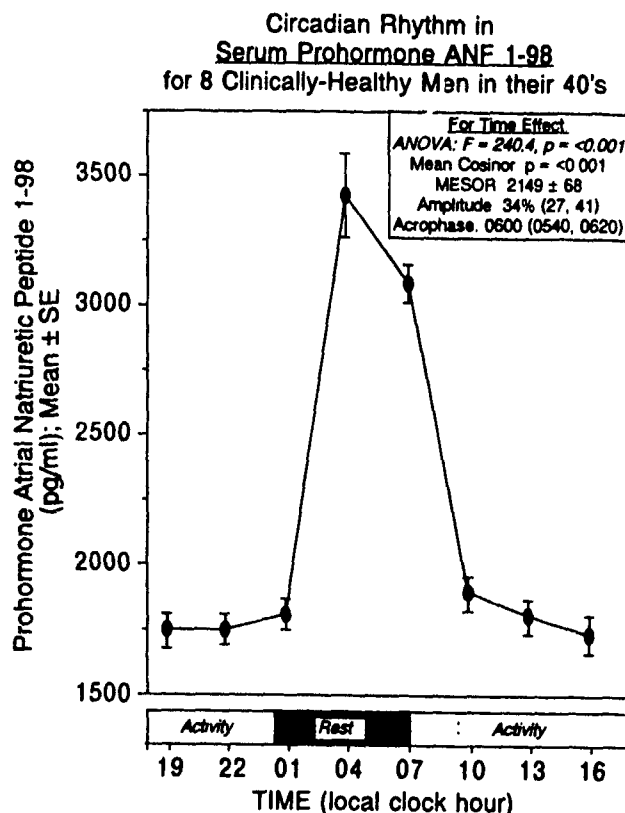
Fig. 1. Mean plasma concentrations (± S.E.M.) of the whole N-terminus (i.e., pro ANF 1-98) of the atrial natriuretic factor prohormone in 8 healthy men in a 24-hour period of normal activity. The peak values at 0400 and 0700 of pro ANF1-98 were significant at P<0.001 compared to all other time points by cosinor analysis.

There have been no other investigations of the N-terminus of the ANF prohormone with respect to circadian variation, but there have been several other studies with respect to ANF and circadian variation. In one study by (Donckier et al.,1986) identical results to the present study were found with the ANF at 0400. One other study (Richards et al., 1987) kept 7 volunteers recumbent for 24-hr and found only a very slight variation of ANF over the 24-hr period. Recumbancy for 24 hr, thus, does appear to affect the circadian peak of ANF that is seen in healthy adults adhering to a more normal pattern of working during the day and sleeping at night.

With respect to blood pressure (b.p.) and these newly discovered b.p. lowering peptides from the heart, mean arterial pressure has been shown to correlate with ANF in a longitudinal study of ANF and b.p. over a 12 week period (McMurray and Vesely, 1989). The N-terminus of the prohormone containing the vasodilatory peptides pro ANF 1-30 and pro ANF 31-67, likewise, correlates closely with mean arterial pressure (McMurray and Vesely, unpublished observation). Infusion of ANF has also been shown to lower b.p. in normotensive and hypertensive individuals (Seymour et al., 1985). The findings of the present investigation that the N-terminus and the C-terminus of the ANF prohormone peak near the normal 24-hour nadir of b.p. (Bristow et al., 1969; Reinberg et al., 1970) may help to explain why blood pressure is lowest at this time of day.

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CORRELATION BETWEEN NEONATAL BLOOD PRESSURE CHARACTERISTICS AND FAMILY HISTORY OF HIGH BLOOD PRESSURE

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ABSTRACT

Starting within two hours after birth, the systolic and diastolic blood pressures (BP) and heart rate (HR) of 72 newborns were automatically monitored at about 30-min intervals for 48 hours with a Nippon Colin (Komaki, Japan) device at the Hospital General de Galicia, Santiago, Spain. On the basis of questionnaires given to the parents inquiring notably about the medical family history, the newborns were assigned a cardiovascular risk score (CRS). This risk scale was obtained according to the presence or absence of cardiovascular disease, elevated BP or obesity across 2 generations, those of the newborn's parents and grandparents. BP and HR values outside ±3 standard deviations (SD) from the individual series mean were removed, and the remaining data were used to compute model dependent (circadian rhythm-adjusted mean or MESOR, amplitude and acrophase) and model independent characteristics (arithmetic mean, SD, 90% and 50% ranges, and circadian range, defined as the difference between the highest and lowest means of hourly classes) for the three circulatory variables. A correlation coefficient was computed between each variable and the CRS. Results indicate highly statistically significant correlations between CRS and indices of variability or dispersion, notably circadian range, SD and 90% range of systolic and diastolic BP and HR, and lack of significant correlation between CRS and average values or indices of location (means, MESORs and even 50% ranges). A 48-hr profile for the computation of extent of change of BP and HR shortly after birth could then provide a neonatal index of cardiovascular risk, to be used for the evaluation of the effects on the newborn of intervention on the pregnant women.

KEY WORDS

Neonates, cardiovascular risk score, blood pressure, heart rate, circadian, correlation coefficient.

INTRODUCTION

Chronobiologic methods (Hermida, 1987) recognize variability as the source of information to be extracted by special hardware and software for use in screening and prevention as well as in diagnosis and cure. Along these lines, rhythm characteristics (as cuantitative endpoints of a component of a biologic time series, formulated algorithmically as a recurrent phenomenon and demonstrated as being periodic by the use of inferential statistics) could complement traditional statistical parameters (e.g., means, ranges and standard deviations) for the detection at birth of the risk of developing a high BP later in life (Halberg et al., 1986; Hermida et al., 1989, 1990). In fact, newborns with a positive family history of high BP have a larger circadian amplitude of BP as compared to newborns with a negative family history (Halberg et al., 1986, 1987).

Neonatal monitoring of BP and HR should be cost-effective irrespective of whether it can improve on or replace inferences drawn from the family history alone. Only a neonatal index for the early

recognition of risk can assess the success or failure of preventive interventions on the pregnant women (such as dietary supplements of calcium and/or magnesium, weight control, etc., to which the family history obviously cannot respond) and the possibly harmful effects (on the baby) of the administration of drugs such as beta-adrenergic agonists or corticoids (Hofman *et al.*, 1983). In that sense, a reduction of the pregnancy-associated high BP (pre-eclampsia) by the administration of calcium has already been reported (Belizan *et al.*, 1983; Kawasaki et al., 1985), but in these studies the timing of calcium intake was not studied, and BP was measured only casually.

SUBJECTS AND METHODS

Within two hours after birth, the systolic and diastolic BP and HR of 72 newborns were automatically monitored at about half-hour intervals for 48 hrs with a Nippon Colin (Komaki, Japan) device at the Hospital General de Galicia, Santiago de Compostela, Spain. On the basis of questionnaires given to the parents inquiring notably about the medical and familial history, the newborns were assigned a CRS. This risk scale was developed on the basis of two principles: 1) presence of overt cardiovascular disease was assigned a risk score twice as large as the risk associated with the presence of an elevated BP, and four times as large as the risk associated with the presence of obesity. And 2) the risk is balanced across 2 generations, those of the newborn's parents and of his grandparents; it is additive within each generation and across generations, with a maximal risk score of 2 per generation. The risk scale thus spans from 0 to 4, with minimum incremental step of .125. Such a CRS is used because even when environmental or other factors may also play a role in the predisposition of the neonate to elevated BP, in the first two days after birth the primary factor for the predisposition to elevated BP later in life comes from heredity (Cornélissen *et al.*, 1989; Hermida *et al.*, 1989, 1990).

BP and HR values outside ±3 SD from the individual series mean were removed, and the remaining data were fitted by linear least-squares with a 24-hour cosine curve (Hermida, 1987). Circadian characteristics (MESOR, amplitude, and linear parameters computed from the amplitude-acrophase pair) and descriptive indices of location and dispersion (mean, 90% range, 50% range, standard deviation and circadian range, defined as the difference between the maximal and minimal hourly mean values after stacking all data to cover a single idealized 24-hr span) for the three circulatory variables were then individually correlated with CRS.

RESULTS

Results (Table 1) indicate highly statistically significant correlations between CRS and indices of variability or dispersion, notably circadian range, SD and 90% range of systolic and diastolic BP and HR, and lack of significant correlation between CRS and average values or indices of location (means,

Table 1: Correlation of blood pressure and heart rate characteristics with a neonatal cardiovascular risk score obtained by questionn··.ie.

Systolic blood pressure			Diastolic blood pressure			Heart rate		
Variable*	r†	P value	Variable*	r†	P value	Variable*	r†	P value
MESOR	-.043	.720	MESOR	-.131	.275	MESOR	.066	.584
A	.345	.003	A	.042	.727	A	.280	.017
A cosØ	.048	.690	A cosØ	-.036	.765	A cosØ	.111	.356
A sinØ	-.213	.072	A sinØ	-.327	.005	A sinØ	.068	.570
Mean	-.010	.936	Mean	-.090	.456	Mean	.051	.669
Stand. dev.	.401	<.001	Stand. dev.	.377	.001	Stand. dev.	.429	<.001
90% range	.417	<.001	90% range	.308	.008	90% range	.430	<.001
50% range	.325	.005	50% range	.195	.101	50% range	.124	.297
Cir. range	.396	<.001	Cir. range	.350	.003	Cir. range	.502	<.001

* MESOR = Circadian midline estimating statistic of rhythm; A = circadian amplitude; Ø = circadian acrophase. MESOR, A and Ø obtained by the least squares fit of a 24-hr cosine curve to data from each individual. Circadian range defined as the difference between the highest and the lowest mean values of hourly classes.
† r = Pearson product-moment correlation coefficient. P = P-value in testing H_0: r=0.

Fig. 1: Correlation between circadian amplitudes (CA) of systolic blood pressure (SBP) and cardiovascular risk score for 72 neonates.

Correlation coefficient r=.345, P=.003
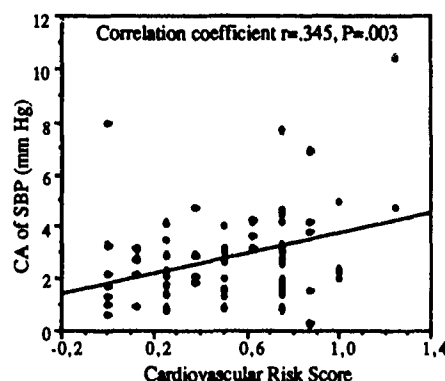
Fig. 2: Correlation between circadian ranges (CR) of systolic blood pressure (SBP) and cardiovascular risk score for 72 neonates.

Correlation coefficient r=.396, P<.001
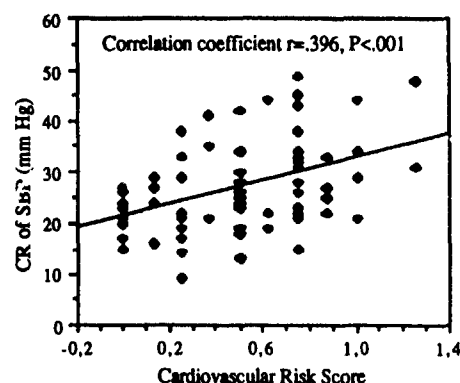
Fig. 3: Correlation between circadian amplitudes (CA) of diastolic blood pressure (DBP) and cardiovascular risk score for 72 neonates.

Correlation coefficient r=.042, P=.727
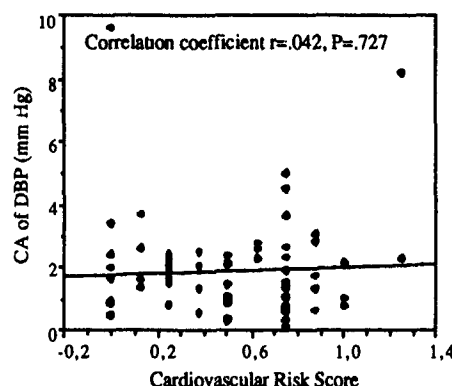
Fig. 4: Correlation between circadian ranges (CR) of diastolic blood pressure (DBP) and cardiovascular risk score for 72 neonates.

Correlation coefficient r=.350, P=.003
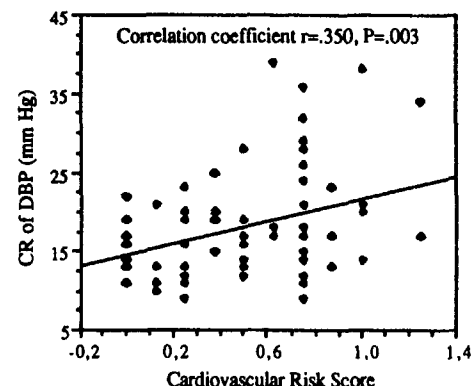
MESORs and even 50% ranges). With respect to the circadian amplitude, the correlation is statistically significant for systolic BP (P=.003, as indicated in Fig. 1) and HR (P=.017, Fig. 5), but not for diastolic BP (P=.727, Fig. 3), even if we remove from analysis either one or both possible outliers characterized by a circadian amplitude larger than 8 mm Hg (Fig. 3). The higher correlations with CRS are obtained with a model-independent index of dispersion, the circadian range. Those correlations are indicated in Table 1 and Figs. 2, 4, and 6 for systolic BP, diastolic BP, and HR, respectively.

DISCUSSION

A 48-hr profile for the computation of extent of change of BP and HR shortly after birth could provide a neonatal index of cardiovascular risk (Halberg et al., 1987; Hermida et al., 1989, 1990), to be used for the evaluation of the effects on the newborn of preventive intervention on the pregnant women (if this index is responsive to such intervention, by contrast to the unalterable CRS obtained according to family history). This index could also need to be correlated with morphologic or functional measures from echocardiography that demonstrate target organ involvement at birth and/or during tracking. In fact, in 14-year-old children, the circadian amplitude of diastolic BP correlates with the interventricular septum thickness, notably in children with a positive family history of high BP (Scarpelli et al., 1987).

Fig. 5: Correlation between circadian amplitudes (CA) of heart rate (HR) and cardiovascular risk score for 72 neonates.
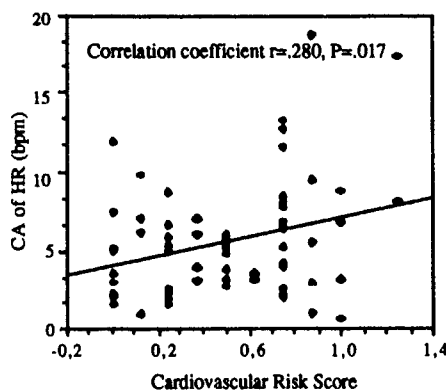
Fig. 6: Correlation between circadian ranges (CR) of heart rate (HR) and cardiovascular risk score for 72 neonates.
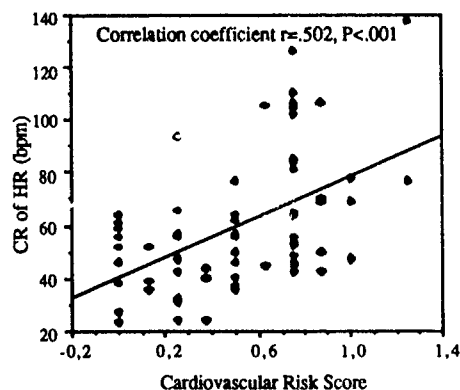
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LINEAR MODEL FOR PREDICTION OF A NEONATAL CARDIOVASCULAR RISK SCORE

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ABSTRACT

The contribution to risk by genetics is a primary factor for the predisposition of a newborn to elevated blood pressure (BP) later in life. An index for this factor is needed to assess, on the neonate, the success or failure of preventive interventions instituted on the pregnant women (such as dietary supplements of calcium and/or magnesium, weight control, etc., to which the family history obviously cannot respond). This index could be based on characteristics of BP and heart rate (HR) variability during the first two days after birth. In the search for such an index, the systolic and diastolic BP and HR of 72 newborns were automatically monitored at about 30-min intervals for 48 hrs with a Nippon Colin (Komaki, Japan) device, starting v.i hin two hours after birth. Circadian chronobiologic characteristics and descriptive statistics foi ,ue three circulatory variables were used in a multiple regression analysis to compute a linear prediction function for a cardiovascular risk score (CRS). This discrete variable was obtained for each neonate on the basis of the presence or absence of overt cardiovascular disease, elevated BP or obesity across 2 generations of his family. Results from regression indicate that the best model includes the circadian amplitudes of systolic and diastolic BP and the circadian range of HR. The linear model was then used to predict the CRS for a new group of 26 neonates. The correlation between the CRS computed from the model and the obtained from the family history of each of those 26 newborns is .918 (P<.001). These results represent a step forward, whereby the effects of possible interventions during pregnancy upon the risk of the development of high BP can be assessed immediately after birth, to indicate those in need for further consideration of primary preventive measures.

KEY WORDS

Neonates, cardiovascular risk score, linear prediction function, multiple regression, circadian, blood pressure, heart rate.

INTRODUCTION

At this point, most of the attempts done to distinguish, at an early age, those persons with an elevated risk for development of high BP from those without such risk have not succeeded. Previous studies have been based mostly on casual BP measurements, not specific in terms of time or rhythm stage (Halberg and Fink, 1981). The chronobiologic approach (using hardware for automatic systematic around-the-clock sampling and software for quantification of rhythm dynamics and regression and/or discriminant analysis of these indices (Hermida *et al.*, 1986)) is the better alternative. By this approach, one can determine a neonatal index of cardiovascular risk that can be used to evaluate the results of preventive measures instituted on the pregnant woman and may provide indications for intervention on the newborn (Hofman *et al.*, 1983), if the index is responsive to measures during pregnancy, by contrast to the unalterable family history (Payne and Hahn, 1986).

Halberg *et al* (1986) already demonstrated a circadian rhythm in BP and HR on an individualized basis for some but not all neonates monitored automatically for 48 hours at 30-minute intervals during the first week of life. Moreover, the circadian amplitude and a linear trend were more prominent in a group of neonates with a positive family history of high BP and cardiovascular disease as compared to a group with a negative such family history (NFH) (Halberg *et al.*, 1985, 1986). We have examined the possibility that differences in rhythm characteristics may be indeed apparent at birth, so that those characteristics can then be used 1) to predict for neonates, from BP readings, the likelihood of high BP in later life, and 2) to test preventive measures that, if validated, could be implemented before actual damage occurs.

SUBJECTS AND METHODS

Within two hours after birth, the systolic and diastolic BP and HR of 72 newborns were automatically monitored at about half-hour intervals for 48 hrs with a Nippon Colin (Komaki, Japan) device at the Hospital General de Galicia, Santiago de Compostela, Spain. On the basis of questionnaires given to the parents inquiring notably about the medical and familial history, the newborns were assigned a CRS. This risk scale was developed on the basis of two principles: 1) presence of overt cardiovascular disease was assigned a risk score twice as large as the risk associated with the presence of an elevated BP, and four times as large as the risk associated with the presence of obesity. And 2) the risk is balanced across 2 generations, those of the newborn's parents and of his grandparents; it is additive within each generation and across generations, with a maximal risk of 2 per generation. The risk scale thus spans from 0 to 4, with minimum incremental step of .125. Such a CRS is used because even when environmental or other factors may also play a role in the predisposition of the neonate to elevated BP, in the first two days after birth the primary factor for the predisposition to elevated BP later in life comes from heredity (Cornélissen *et al.*, 1989; Hermida *et al.*, 1989).

BP and HR values outside ±3 standard deviations from the individual series mean were removed, and the remaining data were fitted by linear least-squares with a 24-hour cosine curve (Halberg *et al.*, 1972). Circadian characteristics (the rhythm-adjusted mean or MESOR and the amplitude) were then used in combination with descriptive indices of dispersion (90% range, 50% range, standard deviation and circadian range, defined as the difference between the maximal and minimal hourly mean values after stacking all data to cover a single idealized 24-hr span) to compute a linear prediction function for CRS by the use of multiple regression analysis (Hermida *et al.*, 1989; Weisberg, 1985).

Three different stepwise procedures were carried out to build a regression model for the neonatal CRS. Methods of stepwise regression use a convenient computational algorithm to limit possible models to a relatively small number, providing a systematic technique for examining only a few subsets of each size (Weisberg, 1985). A path through the possible models is chosen, looking first at a subset of one size, and then looking only at models obtained from preceding ones by adding or deleting variables. The basic algorithms for stepwise regression used here were forward selection, backward elimination and stepwise. In the first procedure, predictors are added at each step. In backward elimination, predictors are eliminated, starting from a model containing all the predictors used for analysis. In the last procedure, a step may be either an addition, an elimination, or an interchange of an "in" variable and an "out" variable (Hermida *et al.*, 1989; Weisberg, 1985).

RESULTS

All three stepwise procedures used to build a regression model for the neonatal CRS concluded with the same result. The model for the prediction of CRS invariably includes the circadian amplitudes of systolic and diastolic BP and the circadian range of HR. The regression coefficients for the model including those three predictors are indicated in Table 1. The coefficient of determination is .8595, indicating that 86% of the variability of the CRS computed from the family history questionnaires is explained by regression on the three predictors. No other variable can be further included in the model since their t statistics are less than the predetermined value (that yielding a P value less than .05 for the number of degrees of freedom considered) to stop the stepwise procedure. The expected average prediction error for the computation of CRS in a new neonate using the linear model in Table 1 is .233.

Figure 1 represents the relation between the CRS computed from the family history questionnaires and the predicted CRS from the linear model indicated in Table 1 for the 72 neonates contributing data in the computation of the model. The multiple correlation coefficient between the predicted and the computed CRSs is .927 (P<.001). The linear model was also used to predict the CRS for a new group

of 26 neonates, monitored according to the same scheme and conditions as the original 72 newborns. The correlation between the CRS computed from the model and the obtained from the family history of each of those 26 newborns is .918 (P<.001), as indicated in Fig. 2. The average absolute prediction error for those 26 neonates was .217, given a P=.663 when compared with the expected value of .233 noted above.

Table 1: Linear model for the prediction of a neonatal cardiovascular risk score obtained by multiple regression analysis on data from 72 Spanish newborns.

Variable*	Estimate	Std. Error	t value	P value
SBP-CA	.14699	2.3815E-2	6.17	<.001
DBP-CA	-.15524	2.9224E-2	-5.31	<.001
HR-CR	.00679	7.5508E-4	8.99	<.001

Residual mean square = 5.4076E-2

Root mean square = .23254

Coefficient of determination (R^2) = .8595

Adjusted R^2 =.8534

* SBP-CA: Circadian amplitude of systolic blood pressure (in mm Hg); DBP-CA: Circadian amplitude of diastolic blood pressure (in mm Hg); HR-CR: Circadian range of heart rate (difference between the highest and the lowest means of hourly classes; in bpm).

Fig. 1: Predicted risk scores from linear model in Table 1 in relation to cardiovascular risk score from questionnaire for 72 Spanish neonates.
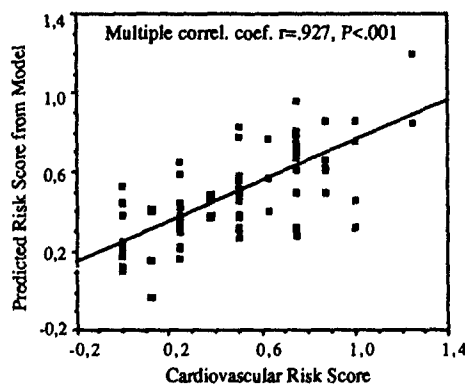
Fig. 2: Correlation between cardiovascular risk score from questionnaire and predicted risk score from linear model in Table 1 for 26 neonates.
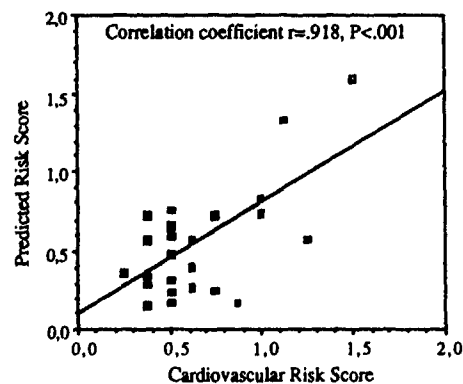
DISCUSSION

Relationships existing between different sets of variables and their relative power with respect to cardiovascular indices at birth were here investigated by methods of multivariate regression analysis (Hermida et al., 1989). A CRS was computed for each neonate and the obtained discrete variable used for building a prediction model based on only three predictors (Table 1). In comparison with similar risk scores used by others (Cornélissen et al., 1989), obesity was now included in the evaluation of the CRS since it is associated with coronary heart disease, primarily because of its influence on BP, blood cholesterol and precipitating diabetes (Payne and Hahn, 1986). Results indicate a high

improvement by the use of the modified risk score. Even when the selected model was the same considering or not obesity as a contributing factor and the estimated regression coefficients were not statistically different between both analyses, the coefficient of determination was improved from .6245 to .8595 when the score for obesity was added in the evaluation of the neonatal CRS.

The tabulated result already represents a first testable battery, even when it may have to be modified as a function of the coexisting risk of diseases other than high BP. The contributions by a paternal versus maternal family history remain to be compared, as are the effects of maternal high BP and various drugs used during pregnancy. Such remaining tasks notwithstanding, the results represent a step forward, whereby the effects of possible interventions during pregnancy (e.g., calcium and/or magnesium supplementation, weight control., etc.) upon the risk of the development of high BP can be assessed immediately after birth, to indicate those in need for further consideration of primary preventive measures.

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SECTION 4

METABOLIC ASPECTS

Chairpersons: H. Decousus and G. Labrecque

TEMPORAL VARIATIONS IN HEPATIC CYTOCHROME P-450 ISOZYMES IN RATS

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ABSTRACT

Temporal variations in hepatic cytochrome P-450 isozymes, and P-450 total
contents and activities were determined by immunochemical quantitation of P-450
isozymes, spectral estimation and measurement of testosterone hydroxylation
activities, respectively, in rats at 4-h intervals. The results indicated that
1) some P-450 isozymes (P-450 UT-2, PB-1, and PB-4&5) had different temporal
variations, 2) several activities of testosterone hydroxylation also had
different temporal variations, and 3) total P-450 contents showed ultradian
change which had 2 peaks. The temporal variation of testosterone hydroxylation
depended on the variation of the P-450 isozyme which efficiently catalyzed each
hydroxylation.   It was concluded that the poor correlation between P-450
activities and total contents was caused by the disparity of temporal variation
of P-450 isozymes which had various catalytic activities, and that each temporal
variation of the P-450 activity depended on the temporal change of the P-450
isozyme having the specificity for the substrate.

KEYWORDS

Temporal variation; cytochrome P-450; P-450 isozyme; testosterone hydroxylase
activity; Western blotting.

INTRODUCTION

It is already known that activities of hepatic cytochrome P-450 show temporal
variations.   However, there is poor correlation between the total contents and
activities of P-450 reported so far concerning temporal variation (Bélanger,
1988). It is considered that this discrepancy will result in different rhythmic
patterns which are caused by various P-450 isozymes (Bélanger, 1988). However,
the temporal changes in P-450 isozymes, have been never reported in the
literature.   In recent years many studies have led to the isolation,
purification and characterization of many P-450 isozymes (Gonzalez, 1989).
Particularly, Funae and Imaoka(1985, 1987), and Imaoka et al. (1987a, 1987b)
purified the most numerous isozymes of P-450 from rats, and that advancement of
the investigation on P-450 isozymes 'ed to this study.

The purpose of this study is to investigate temporal variation in the different

P-450 isozymes and activities, and clarify the reason for the discrepancy
between the contents and activities of P-450. We measured 1) the content of P-
450 isozymes by Western blotting, 2) total P-450 content, and 3) testostrone
hydroxylase activities in rat hepatic microsomes at 4-h intervals.

MATERIALS AND METHODS

Male Wistar rats, 8 weeks-old, were used. Rats were kept in an animal room
illuminated from 0800h to 2000h for 2 weeks at 25 °C and sacrificed at 4-h
intervals at 1600h, 2000h, 2400h, 0400h, 0800h, 1200h and 1600h. Each group
comprised 4 animals. Rat liver microsomes were prepared as described in a
previous report (Funae and Imaoka, 1985). Immunochemical quantitation of P450
UT-2(UT-A, RLM5, P-450h, P-450-male and P-450 2c by other researchers), PB-1(not
reported by others) and PB-4&5(P-450-b&e) by immunoblotting was done as
described previously (Imaoka et al., 1987a). Antibody against purified P-450
was prepared using female Japanese rabbit (Imaoka et al., 1987a). Total P-450
contents were estimated spectrally by the method of Omura and Sato (1964).
Testosterone hydroxylation activity was measured by a method described before
(Imaoka et al., 1989). The hydroxylated testosterone standards 2α-, 16α-,
6β- and 16β-hydroxytestosterone were kinding donated by Dr. Y. Nakamura, and
2β-hydroxytestosterne was provided by Prof. D. N. Kirk. The protein
concentration was measured by the method of Lowry et al (1951). For statistical
analyses, analysis of variance (one way ANOVA) and Student's t-test were used.

RESULTS

As shown in Fig.1A, P-450 UT-2 and PB-1 indicated ultradian variation having two
peaks. The peak values of P450 UT-2 and PB-1 were obtained at 0800h and 2000h,
and at 1200h and 2400h, respectively. The ratio of the peak and the trough
value of P450 UT-2 and PB-1 were 1.5 and 2.1, respectively. P450 PB-4&5 showed
circadian variation having the peak at 2400h, and the ratio of the peak and the
trough value was 2.5. Total P-450 contents showed ultradian variation which had
two peaks at 0800h and 2000h. Three types of temporal variation of
testosterone hydroxylation activity were observed as shown in Fig.1B. The two
peak values for testosterone 2α- and 16α-hydroxylation were observed at 0800h
and 2000h. The two peak values for 2β- and 6β-hydroxylation were obtained at
1200h and 2400h. 16β-hydroxylation activity did not indicate temporal
variation. At 2400h, 2α- and 16α-hydroxylation, and 2β- and 6β-
hydroxylation of testosterone showed minimum and maximum activity, respectively.
The differences between the peak value and the trough value were significant
($p<0.05$, Student's t-test) for all P-450 isozymes and activities, except for
testosterone 16β-hydroxylation activity. Temporal variations were significant
for P450 UT-2 ($p<0.005$, ANOVA), PB-1 ($p<0.005$), PB-4&5 ($p<0.005$), testosterone
2α-hydroxylation activity ($p<0.005$), and 15α-hydroxylation activity ($p<0.05$).

DISCUSSION

There were 4 types of temporal variations in P-450 isozymes and testosterone
hydroxylation activities. First, P450 UT-2 showed ultradian variation which had
two peaks at 0800h and 2000h. The variation was similar to temporal change of
testosterone 2α- and 16α-hydroxylation having two peaks at 0800h and 2000h.
It is known that 2α- and 16α-hydroxylation of testosterone is mainly catalyzed
by UT-2 and UT-3 (Funae and Imaoka, 1987). Total P-450 contents also had a
similar temporal change. Second, P450 PB-1 indicated ultradian variation, which
had the two peaks at 1200h and 2400h. The rhythmic pattern was similar to
variation of 2β- and 6β-testosterone hydroxylation (having the two peaks at
1200h and 2400h), efficiently catalyzed by P450 PB-1 (Imaoka et al., 1988).
Third, P450 PB-4&5 indicated circadian variation which had the peak value at
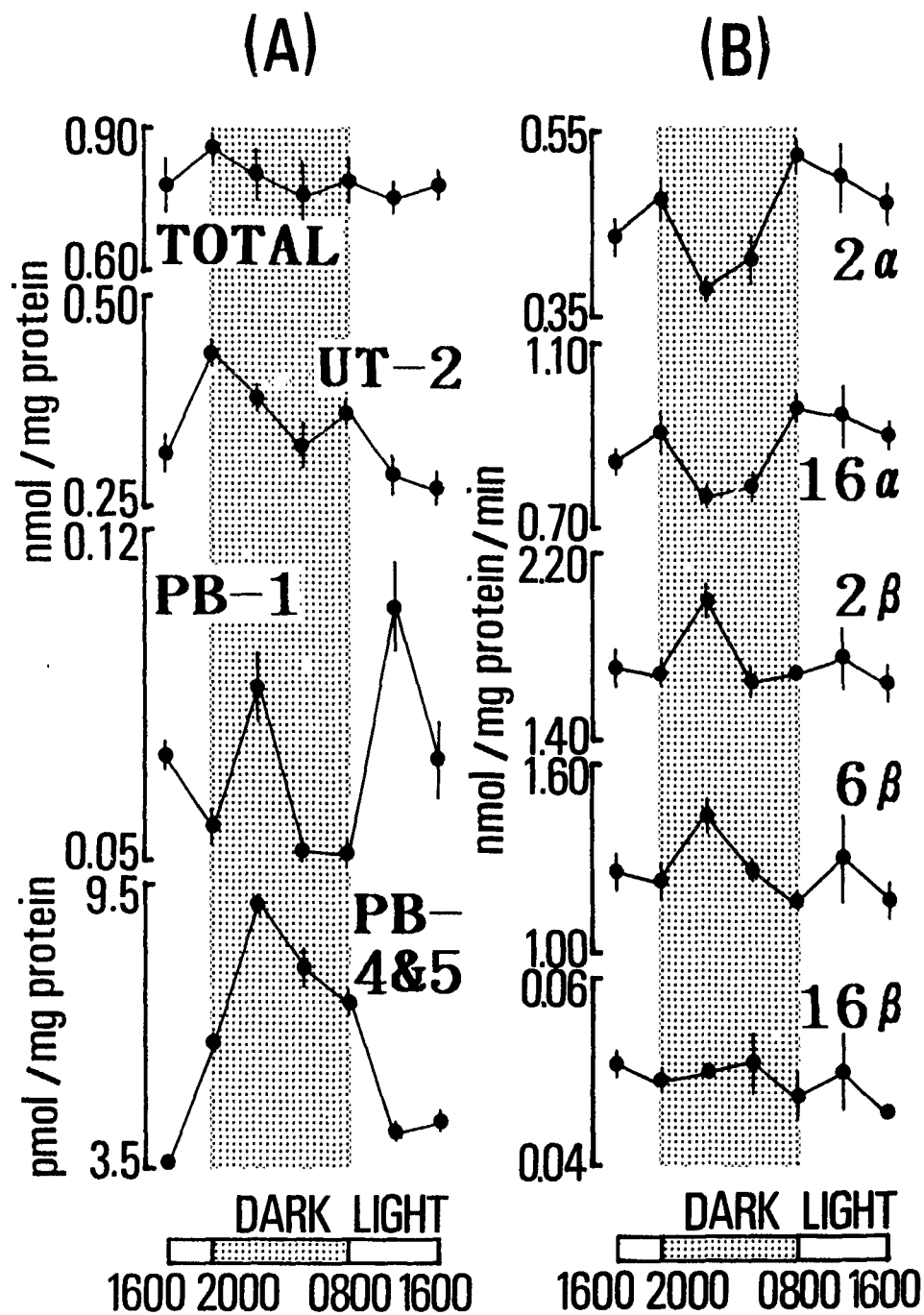
Fig. 1. Temporal variations of total P-450 contents, P450 UT-2, PB-1 and PB-4&5 (A), and 2α-, 16α-, 2β-, 6β- and 16β-hydroxylation of testosterone (B) in rat liver microsomes. Vertical bars represent the Standard Error.

2400h.   Fourth, testosterone 16β-hydroxylation activity did not show the temporal change.

The temporal change in the activity of testosterone hydroxylation corresponded to the change in the P-450 isozyme which efficiently catalyzed each hydroxylation.   It is known that testosterone hydroxylase activity reflects the distribution of isozymes of P-450 (Funae and Imaoka, 1985, 1987; Imaoka *et al.*, 1987a, 1987b,1988 and 1989).   With respect to total P-450 contents, the ultradian variation of the contents having two peaks, strongly depended on the temporal variation of P450 UT-2, which was a maximal constitutive form in male rats.   Although there are many reports about the different rhythmic patterns of P-450 activities at the present time, it is natural that disparity exists between these reports.   Since these chemicals are catalyzed by one or more isozymes and these isozymes have different rhythmic patterns, P-450 activities have various temporal variation.   On the other hand, since total P-450 content corresponds to the sum of many P-450 isozymes, there is also poor correlation between the total P-450 contents and activities.

In conclusion, it was elucidated that the poor correlation between activities and total contents of P-450 was caused by the different temporal variations of many P-450 isozymes which had various catalytic activity, and that each temporal variation of the P-450 activity depended on the chronobiological change of each P-450 isozyme having the specificity for the substrate.

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CIRCADIAN VARIATIONS IN PLATELET FUNCTIONS AND BLOOD COAGULATION PARAMETERS

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ABSTRACT

Platelet aggregation in response to adenosine diphosphate (ADP) and adrenalin (A), platelet adhesiveness in a glass bead column, prothrombin time (PT), activated partial thromboplastin time (PTT), thrombin time (TT), fibrinogen, Factor VIII activity, and alpha-1-antitrypsin antigen showed circadian rhythms verified by cosinor analysis in 10 clinically healthy diurnally active subjects studied at six circadian times in a latin square design. The timing of these rhythms may determine temporary risk states for bleeding episodes and/or thromboembolic phenomena.

KEYWORDS

Circadian variations, coagulation parameters, platelet retention, platelet aggregation (adrenalin, ADP).

INTRODUCTION

Circadian periodicity characterizes the incidence of myocardial infarction (Master and Jaffee, 1952; Reinberg et al., 1973; Muller et al., 1985) sudden cardiac death (Muller et al., 1987; Rabkin et al., 1980) cerebral infarction (Reinberg et al., 1973; Marshall, 1977; Marler et al., 1989), and acute intracerebral hemorrhage (Ramirez-Lassepas et al., 1990). The circadian periodicity in the occurrence of these conditions suggests the existence of transient rhythmic and thus predictable risk states for acute and catastrophic events related to thrombosis and hemorrhage. Reports by numerous investigators have described circadian rhythms in parameters related to blood coagulation (Petralito et al., 1982; Foruasari et al., 1977) fibrinolysis (Grimando et al., 1988; Huber et al., 1988), and effects of anticoagulants, i.e. heparin (Decousus et al., 1985). Also circadian rhythms in numbers of circulating platelets (Haus et al., 1983) and platelet aggregability (Tofler et al., 1987; Petralito et al., 1982) have been described. It appeared of interest to study platelet aggregability and in addition, platelet adhesiveness and a battery of coagulation related parameters, together with some well documented circadian rhythms like plasma cortisol and the number of circulating white blood cells as biologic time reference in the same clinically healthy subjects.

SUBJECTS AND METHODS

Ten clinically healthy subjects (5 men and 5 women) 31 $\pm$ 11 (1SD) years of age (range 21-50) following a diurnal activity pattern with rest at night (on the average from 2330 to 0630) and eating a regular 3 meal diet were studied at six time points (0800, 1200, 1600, 2000, 0000, and 0400) spread in a latin square design over a 1 week span during August. Except for 30 minutes prior to blood drawing and during the night hours, the subjects were ambulatory. Blood was drawn in recumbent position with minimal tourniquet action. Platelet rich plasma was obtained by centrifugation at 500 RPM and adjusted to 300,000 platelets per ml. Platelet aggregation in response to ADP and adrenalin was measured by a decrease in optical density in a Biodata aggregometer (PAP-2A). Platelet retention on a glass bead column was measured in six 1 ml aliquots of each sample with automatic injection. The platelets were counted manually by the same two experienced technologists in duplicate in a Neubauer bright line chamber.

Prothrombin time (PT) was performed by the one stage method using Dade liquid activated thromboplastin and 0.015 M $CaCl_2$. Activated partial thromboplastin times (PTT) were performed with General Diagnostics automated APTT reagents. Fibrinogen was measured by the functional assay using Dade reagents. Factor V and VIII coagulant activity were measured in citrated plasma using one state clotting factor assays based on the prothrombin time (factor V) and the APTT (factor VIII) with deficient plasma obtained from human donors. Coagulation endpoints were measured on a BBL Fibrometer and a Biodata Coagulation Profiler.

Antithrombin III antigen, plasminogen antigen, alpha-2-macroglobulin antigen and alpha-1-antitrypsin antigen were determined by radial immunodiffusion using Behring Diagnostic reagents and equipment.

A complete blood count (Coulter S), differential (200 cell) count and plasma cortisol (by RIA) were determined as reference functions. The data obtained were analyzed by the cosinor technique, (Nelson et al., 1979) and/or by one way ANOVA and peak-trough differences were confirmed by T-test.

RESULTS AND DISCUSSION

Platelet aggregation in response to adenosine diphosphate (ADP) and to adrenalin (A) showed a circadian rhythm with peak (minimal OD) during the night hours (0000) and a trough (maximal OD remaining) at 1600 (Fig. 1). The cosinor summary is shown in Table 1. This timing is at variance with the findings reported by Tofler et al. (1987) who found a rise in aggregability in response to ADP and adrenalin between 0600 and 0900, with peak at 0900 and the lowest values between 0300 and 0600. We do not have an explanation for this difference at the present time. Some differences in the endpoints measured and/or different stages of infradian rhythms (e.g. circaseptan or circannual) may have to be considered (Imeson et al., 1987).

In contrast, platelet adhesiveness as measured by retention in a glass bead column showed a large amplitude circadian rhythm with a peak at 0800 and a trough at midnight (Fig. 1). This timing would correspond to the reported peak-incidence in the onset of myocardial infarction and slightly precede that of sudden cardiac death.

PT, PTT and TT showed a circadian rhythm with a statistically significant differences in acrophase validated by the Bingham parameter test. TT and fibrinogen show a morning peak as does Factor VIII activity. Alpha-1-antitrypsin shows the acrophase at 1000 with 95% confidence interval between 0820 and 1136 suggesting an increase in fibrinolytic activity during daytime.

The plasma concentrations of plasminogen, α-2-macroglobulin, antithrombin III,

Factor V and fibrinogen degradation products showed a high degree of
variability, which did not allow rhythm detection by cosinor. Plasma cortisol
and the hematologic reference functions showed statistically significant
circadian periodicity with the acrophase at the expected time confirming the
circadian synchronization of the subjects.
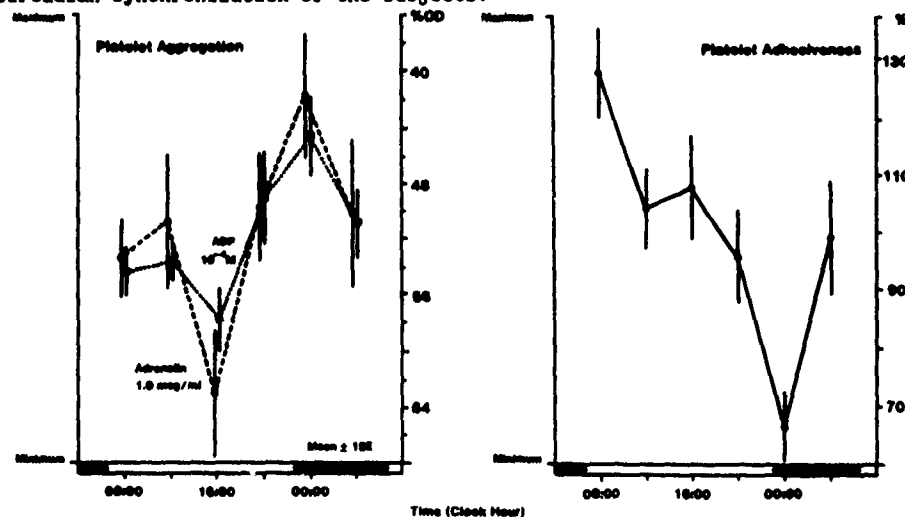
Fig. 1 Circadian rhythm in platelet aggregation (after stimulation with
adenosine diphosphate and adrenalin) and in platelet retention (adhesiveness) on
a glass bead column in 10 clinically healthy subjects.

Table 1. Cosinor summary of coagulation parameters and reference
functions in 10 clinically healthy subjects.

Parameter	p	Mesor M	±1SE	Amplitude A	95%CI	Acrophase ø	95%CI
Platelet Aggregation							
Adrenalin (OD at Completion)	0 022	51.43	2 84	6 34	1.03 11.86	01 24	20 48 05 40
ADP 10^-4 M (OD at Completion)	0.029	52.52	1 76	3 95	0 39 8 68	01·04	22.16 07 44
Platelet Retention (% of Mean) (Glass Bead Retention)	0.001	100 01	4 09	20 30	10 53 30 27	10·44	08.16 13:20
Prothrombin Time	0 004	10 30	0 11	0 28	0 11 0.47	16·28	13 52 19:56
APTT	0.004	29.31	0 68	1.18	0.45 1.92	22.16	18:44 01 40
Thrombin Time	0.007	14.62	0 25	0.26	0.09 0 44	04.28	01.00 07 36
Fibrinogen	0.001	226.85	.3 43	11 45	7.30 17 36	08·28	06 08 11·40
Alpha-1-Antitrypsin	0.001	208.56	10.37	20 39	13.01 27.78	10.00	08:20 11:36
Facor VIII	0.038	160.25	15 67	11 45	0.69 22 23	08 20	03.56 13 00
Plasma Cortisol	0 001	10 67	0.59	6 42	4.87 8.32	07·48	06.28 09:32
Neutrophils, Circulating	0.017	3032	273	294	60 538	18 24	14.20 22·48
Lymphocytes, Circulating	0.045	2253	197	316	7 628	22:44	17·28 04 16
Eosinophils, Circulating	0.018	226	18	65	12 120	22 52	20 20 02.36

These findings confirm and extend the observations on a circadian time structure
of the coagulation and fibrinolytic system. There are important phase
differences between some of the coagulation parameters which may be of interest
for the pathophysiology of blood coagulation and related disorders. The
circadian variations in different aspects of platelet function, coagulation and
fibrinolytic parameters in a diurnally active circadian synchronized population
of clinically healthy subjects suggest time dependent differences in the complex
blood coagulation mechanisms, which may indicate temporary risk states for
bleeding episodes and/or thrombosis, which may be predictable in their timing.

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CAN PLASMA LEVELS OF *S*- AND *R*-WARFARIN BE CORRELATED WITH THE DRUG-INDUCED ACCUMULATION OF VITAMIN K EPOXIDE IN RAT LIVER MICROSOMES?

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ABSTRACT

The objective of this research was to determine whether a time-related correlation can be made between plasma levels of warfarin's isomers and the liver activity of vitamin K 2,3, epoxidase in liver microsomes prepared from control and warfarin-treated rats. The data indicated that the ratio of S-/R-warfarin plasma levels and the warfarin-induced inhibition of the epoxidase activity were both maximal at 05h00 and minimal 12 hours later. This is the the same hours of day of warfarin's strongest and smallest inhibitory action of blood coagulation. Thus, the time-dependent variations in the anticoagulant action of warfarin can be explained in part by the temporal changes in the kinetics of the drug and by its inhibitory action on liver 2,3 epoxidase.

KEYWORDS

Warfarin enantiomers, liver microsomes, vitamin K 2,3 epoxide cycle, rat.

INTRODUCTION

The major pharmacological effect of warfarin is the inhibition of blood clotting by interference with the Vitamin K cycle in the liver. Specifically, warfarin inhibits the activity of the 2,3 epoxide reductase (Whilton et al., 1978) and the vitamin K reductase (Fasco and Principe, 1982).

It is interesting to note that the pharmaceutical preparation of warfarin administered to patients is a racemic mixture but the anticoagulant action of S-warfarin is 2-5 times more potent than that of R-warfarin in rat (Yacobi and Levy, 1974). Furthermore, we showed recently that the anticoagulant effect of warfarin was dependent on the hour of its administration (Soulban and Labrecque, 1988). The objective of the present work was to

determine the mechanism of this time-dependent variation in the effect of warfarin by examining whether time-related correlations can be established between plasma concentrations of the 2 enantiomers and the inhibitory action of warfarin on the activity of microsomal vitamir K 2,3 epoxide.

METHODS

Adult male Spague-Dawley rats (150-250g) were housed 2 per cage in a temperature controlled room (23 ± 2 °C) on a 12 hr light-dark cycle (light on: 06h00-18h00) for at least 2 weeks prior to experimentation. Purina Lab Chow and water were available *ad libitum.*

Groups of 5 rats received orally either a dose of 500 µg/kg of racemic sodium warfarin or an equal volume of 0.9% NaCl at 01h00, 05h00, 09h00, 1ɔh00, 17h00 and 21h00. Twenty-four hours after drug or saline administration, rats were anesthetized with halothane, blood was collected by cardiac puncture, the livers were removed and the hepatic microsomes were prepared. Plasma levels of warfarin was determined as described elsewhere (Soulban et al., 1990). The activity of vitamin K epoxide was determined according to Wallin and Martin (1987). Briefly, a final concentration cf 0.1mM of vitamin K epoxide was added to 0.5 ml of microsomal preparation and the reaction was stopped after a 30 min incubation. The concentration of vitamin K epoxide was determined spectrophometrically and the differences in the epoxide levels in control and warfarin-treated animals was used as an indirect index of enzymatic activity. The data was subjected to linear regression analysis and the two tailed Student's t Test was used in the in vitro experiments. A $p < .05$ was considered to indicate statistical significance.

RESULTS

Table 1 presents the plasma concentrations of S- and R-warfarin found 24-hr after administration of the anticoagulant at 5 times of the day. No significant differenc? could be detected between these 24-hr values but the ratio of S-/R-warfarin plasma concentrations at 05h00 (2.12 ± 0.25) was significantly higher that the S/R ratio obtained at 01h00 (1.19 ± 0.22) or at 21:00 (0.99 ± 0.19).

In microsomes prepared from control rats (hatched bar), Figure 1 illustrate that the lowest concentration of Vitamin K epoxide (0.62 ± .39 µg/mg) was obtained at 09h00 while the highest level (8.80 ± 2.4 Mg/mg) was found between 21h00 and 01h00. These data suggest that the activity of vitamin K epoxide was largest at 09h00 and much smaller 12 hrs later.

Figure 1 indicates also that warfarin administration at 05h00 increased significantly (+356.3%) the microsomal levels of vitamin I, epoxide which rose from 1.6 ± 0.1 µg/mg in controls (hatched bar) to 5.7 ± .97 µg/mg in warfarin-treated microsomes (black bar). It is interesting to note that no significant change could be detected when warfarin was administered at the 5 other times of day.

TABLE 1

TIME-RELATED CHANGES IN S- AND R-WARFARIN LEVELS IN RAT PLASMA.

Hour of the day	Plasma levels[1]		S-/R-Warfarin ratio
	S-Warfarin	R-Warfarin	
07h00	0.37 ±.10	0.40 ±.12	1.19 ±.22*[2]
05h00	0.51 ±.13	0.27 ±.11	2.12 ±.25*
13h00	0.39 ±.04	0.24 ±.03	1.64 ±.19
17h00	0.47 ±.14	0.30 ±.07	1.56 ±.19
21h00	0.23 ±.05	0.19 ±.03	0.99 ±.19*

1. The plasma levels were obtained 24 hrs after oral warfarin administration; they are expressed in µg of the enantiomers/ml of plasma.
2. The asterisk indicates a statistically significant difference ($p < .05$) in the 05h00 S/R warfarin ratio and those obtained at 01h00 and 21h00.
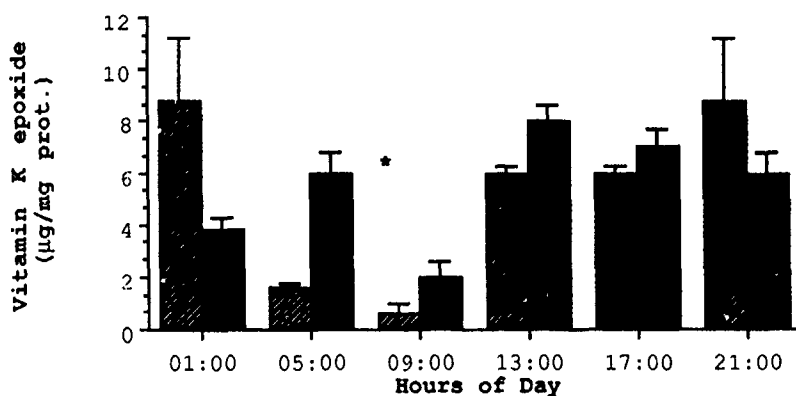
FIGURE 1: TIME-DEPENDENT ACCUMULATION OF VITAMIN K 2,3 EPOXIDE IN LIVER MICROSOMES OF RATS. In microsomes prepared from control (hatched bars) or warfarin-treated (black bars) rats, vitamin K epoxide was incubated and the epoxide levels was determined at the end of the incubation time. The asterisk indicates a statistically significant difference ($p < .05$) in epoxide levels obtained in the 2 groups of animals.

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DISCUSSION

The time-dependent variations in the anticoagulant effect of
warfarin (Soulban and Labrecque, 1988) could be related to the
fact that the plasma levels of the drug or the enzymatic activity
of vitamin K epoxide reductase and vitamin K reductase or the
drug-induced inhibition of these enzymes were not constant
throughout the day. Our *in vitro* studies with vitamin K 2,3
epoxide support this hypothesis because the data indicate that the
levels of epoxide in control microsomes were significantly smaller
($P<.05$) at 05h00 and 09h00 than at any other moments of the day.
In warfarin-treated rats, maximal accumulation of epoxide was
found at 05h00 in microsomes. Thus, it is interesting to note
that maximal inhibition of the epoxidase activity, the highest
ratio of S-/R-warfarin plasma levels that are both reported above
as well as the maximal anticoagulant action of warfarin (Soulban
and Labrecque, 1988) were all obtained at the same time of the
day, i.e. 05h00. However, further research is needed to identify
completely the mechanism of time-dependent variation in the
anticoagulant effect of warfarin because vitamin K reductase is
now thought to be involved in the warfarin's effect (Fasco and
Principle, 1982; Preusch and Suttie, 1984). No data are available
to date on the circadian variations in the activity of this enzyme
and in the effect of warfarin on the activity of this enzyme.

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TEMPORAL VARIATIONS IN THE PLASMA CONCENTRATIONS OF *S*- AND *R*-WARFARIN IN THE RAT

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ABSTRACT

To determine whether time-dependent variations could be detected in the plasma concentrations of S- and R-warfarin, 2 groups of rats received 500 µg/kg of racemic warfarin (p.o) at 06h00 and 18h00 and blood was withdrawn up to 24 hours after warfarin administration. At 06h00, maximal concentrations of S- and R-enantiomers occurred 6-8 hours after warfarin administration; at 18h00, maximal concentrations were obtained 2-6 hours after drug administration. At the 2 time points studied, the $t_{1/2}$ of S-warfarin was always longer than for R-warfarin and the $t_{1/2}$ of both enantiomers was always smaller during the activity period of the animals.

KEYWORDS

Plasma levels, S-warfarin, R-warfarin, temporal variation, rat.

INTRODUCTION

Studies in human have shown S-warfarin to be 2-5 times a more potent anticoagulant than R-warfarin (O'Reilley, 1974; Wingard et al., 1987) and in animal (Yacobi and Levy, 1974). Recently, we reported (Soulban et al., 1988) the time-dependent pharmacological action of warfarin: factor II activity was significantly more inhibited by warfarin at 05h00 compared to 21h00. The time of maximal inhibition of factor activity corresponded to the same time of day when there was a higher plasma S/R enantiomeric ratio

(Soulban et al., 1990). This finding raised a new question: do the plasma levels of S- and R-warfarin vary differently as a function of time of warfarin administration?

METHODS

Adult male Spague-Dawley rats (150-250g) were housed 2 per cage in a temperature controlled room (23 ± 2 ºC) on a 12 hr light-dark cycle (light on 06h00-18h00) for at least 2 weeks prior to experimentation. Purina Lab Chow and water were available *ad libitum*.

The rats were divided into 2 groups, one received 500 µg/kg of racemic sodium warfarin orally at 06h00 and the second at 18h00. Rats were anesthetized with halothane and blood was collected by cardiac puncture at 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0 and 24.0 hours after warfarin administration. Blood was diluted in a proportion of 9 parts of blood to one part of 3.1% sodium citrate. Plasma was obtained and frozen at -80 ºC until analysis.

The separation and quantification of warfarin enantiomers was done according to Banfield and Rowland (1984). Warfarin was extracted from plasma samples and dried to completeness, afterwhich the samples were derivitized to form an ester. The esterified R- and S-warfarin were separated by h.p.l.c. on a silica column and then the ester moieties were cleaved by a post-column reagent (n-butylamine-methanol) on a glass bead column. The isomers and standard (7-ethoxycoumarin) were detected by fluorescence. Quantification of plasma concentrations was based on calibration curves which used peak height ratios of each isomer to the reference standard.

RESULTS

Table 1 presents the time-dependent changes in S- and R-warfarin plasma levels after warfarin administration at 2 times of the day. After the 06h00 warfarin administration, S- and R-warfarin reached maximal concentrations of 1.32 ± 0.35 and 0.44 ± 0.18 µg/ml, respectively, at 8.0 hours. After the 18h00 administration, maximal levels of 1.71 ± 0.28 µg/ml for S-warfarin and 0.53 ± 0.13 µg/ml for R-warfarin were attained between ^ 0-6.0 hours. Maximal concentrations of S- and R-warfarin occurred earlier and were

TABLE 1

ANALYSIS OF WARFARIN ENANTIOMERS IN RAT PLASMA AT 2 HOURS OF DAY

Hours after administration	S-Warfarin levels[1]		R-Warfarin levels[1]	
	06h00	18h00	06h00	18h00
0.5	0.82 ±.02	1.08 ±.08	0 17 ±.01	0.30 ±.06
1.0	0.73 ±.06	0.88 ±.18	0.19 ±.04	0.25 ±.04
2.0	1.02 ±.13	1.70 ±.28	0.25 ±.06	0.50 ±.12
4.0	1.03 ±.12	1.54 ±.10	0.29 ±.04	0.51 ±.11
6.0	1.22 ±.22	1.71 ±.28	0.37 ±.11	0.53 ±.13
8.0	1.32 ±.25	1.35 ±.11	0.44 ±.13	0.40 ±.04
12.0	1.01 ±.16	1.12 ±.04	0.27 ±.04	0.27 ±.01
24.0	0.80 ±.08	1.10 ±.23	0.21 ±.04	0.34 ±.11

1. The plasma levels of S- and R-warfarin are expressed as $\mu g/ml$ ± SE. Each value is mean levels obtained in 3 rats/time points.

statistically greater at 18h00 when compared to the 06h00 values. Also, 1.0, 2.0, 4.0 and 6.0 hours after 06:00 and 18:00 warfarin administrations, the plasma concentrations of both enantiomers were statistically different than 8.0, 12.0, 24.0 and 0.5 hours after drug administration. It is interesting to note that $t_{1/2}$ of S-warfarin was calculated to be 48.5 hrs after the 06h00 administration and 32.8 hrs at 18:00. In the case of R-warfarin, the t1/2 was also smaller during the activity period of the animal as it was found to be 41.2 hrs at 06h00 and 29,5 hrs at 18h00.

DISCUSSION

We have reported time-dependent changes in the anticoagulant effect of warfarin (Soulban and Labrecque, 1988) in the rat. As it was the case for heparin in human (Decousus et al., 1985), the maximal effect of both anticoagular 3 in rats occurs at 04h00 or 05h00. However, heparin and warfarin are producing their anticoagulant effect by different mechanisms and the pharmaceutical preparation of warfarin is a racemic mixture which acts like two separate drugs with different pharmacodynamic and pharmacokinetic properties; S-warfarin is 2-5 times more potent than R-warfarin in rat (Yacobi and Levy, 1974). The present study

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not only confirms that S- and R-warfarin have different
pharmacokinetic profile but that these properties are dependent
upon the time of warfarin administration. S-warfarin had a
shorter half-life and was more rapidly eliminated at 18h00
(activity period) while R-warfarin also had a shorter half-life at
18h00 but was eliminated more slowly compared to 06h00. The fact
that the R- isomer was eliminated more rapidly than the S- isomer
is confirmed by other investigators (Breckenridge and Orme, 1972;
Yacobi and Levy, 1974). Interestingly, the data that show both
enantiomers to have shorter half-lives during the dark period is
further confirmed by other results (Soulban et al., 1990). The
lowest S/R-warfarin plasma ratio occurred at 21h00 and 01h00 which
was the same time period when warfarin's inhibitory action on its
target site, the vitamin K 2,3 epoxide reductase, was minimal.

The light/dark time periods probably influence the
pharmacokinetics of each enantiomer at the level of metabolism and
protein binding. Warfarin has a low hepatic extraction ratio, is
highly metabolized by the cytochrome P-450, is highly protein
bound (98-99%) and the clearance of each enantiomer is a function
of their free fraction (Breckenridge and Orme, 1972; Yacobi and
Levy, 1974). Temporal variations may occur at any or all of these
levels.

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MORNING HYPERCOAGULABILITY IN MAN

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KEYWORDS

circadian plasma fibrinogen,arterial thrombosis,coagulation system,heparin.

INTRODUCTION

Arterial thrombosis appears to be a common and essential link in the onset of most cases of acute vascular events (1). Thus, it is not surprising that these vascular disorders and the coagulation processes are correlated. For example, high levels of plasma fibrinogen were associated with an increased risk of myocardial infarction (2). On the other hand a circadian variation has been demonstrated in the incidence of myocardial infarction (3), sudden cardiac death (4) and thrombotic stroke (5) with a morning peak and a nocturnal trough. One of the mechanisms of these variations might be a circadian variation in coagulation processes which might also explain the circadian rhythm observed in anticoagulant effect of heparin (6). To evaluate such hypothesis we have measured, in healthy volunteers, plasma fibrinogen and activated partial thromboplastin time (APTT), every 3 hours for 24 hours.

SUBJECTS AND METHODS

. Subjets :

Eight healthy volunteers participated in this study, performed in spring (April). Their informed consent was obtained and the protocol was approved by the local ethic committee. Six were men and two women. Their mean age was 25 (range 23 - 29) years. All subjects were synchronised by daily activity (07.00 - 23.00) and nocturnal sleep (23.00 - 07.00). Meals were taken at fixed times (07.00, 12.00 and 19.00).

. Coagulation tests :

Veinous blood was taken at 06.00 and then every hours for 24 hours. For each blood sample five millitres were collected in polystyrene tubes (nine volumes of blood and one volume of 0.129 M trisodium citrate). After being collected each blood sample was centrifuged for 20 minutes at 2500 gr at 4°C and stored frozen at - 80° C until assayed. All plasma were tested within a week after the blood collection immediately after thawing at 37°C. Assay for APTT was performed using the same reagent (AUTO APTT reagent, general Diagnostics Laboratories) and a clot detecting device (Coagamate X2). APTT was expressed in seconds. Assay for plasma fibrinogen was performed with an

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other clot detecting device (AUTO-FI) according to the method of Von Clauss (7). Plasma fibrinogen levels were expressed in gramm per litre. All measurements were performed in blind without the knowledge of the sampling hour.

. Statistical analysis :

Data were analysed by both conventional and cosinor methods. The analyses were performed on raw values and data transformed as percentages of the individual 24 hour mean.

RESULTS

. Plasma fibrinogen :

Whether the mean values for the eight volunteers were expressed as raw values (Table I) or as percentages of the individual 24 hours mean (Figure 1) plasma levels of fibrinogen exhibited a large circadian variation with a morning peak and a nocturnal trough. Differences between peak and trough reached almost 20 % (Figure 1). For raw values cosinor analysis validated a circadian rhythm (p ☐ 0.05). In addition, for data expressed as percentages of the individual 24 hour mean, two ultradian rhythms with periods of 12 hours and 8 hours were also validated by cosinor method (p<0.05).

. Activated partial thromboplastin time :

The mean values for the eight volunteers, expressed as raw values (Table I) and as percentages of the individual 24 hour mean (Figure 1), exhibited a opposite time course. There was a morning trough and a nocturnal peak. However the amplitude of this variation was smaller than that observed for plasma fibrinogen. Cosinor analysis validated a circadian rhythm only for data expressed as percentages of the individual 24 hours mean (P<0.01). Two ultradian rhythms with periods of 12 hours and 8 hours were also validated (p<0.05).

Table I - Mean values (±S D.) of activated partial thromboplastin time (A P T T) expressed in seconds and plasma fibrinogen expressed in gramme / litre (g/l), measured every 3 hours for 24 hours in eight healthy volunteers

COAGULATION PARAMETERS

SAMPLING TIME (Hours)	A P T T. (seconds)	PLASMA FIBRINOGEN (g/l)
08	29 7 ± 1 8	2 66 ± 0 74
11	29 3 ± 2 1	2 93 ± 0 75
14	30 0 ± 2 3	2.92 ± 0 77
17	30 8 ± 3 4	2.88 ± 0 76
20	29 7 ± 1.8	2 68 ± 0 80
23	30 5 ± 2.3	2 75 ± 0 80
02	32.2 ± 2.7	2 52 ± 0 69
05	31 0 ± 2 3	2 45 ± 0 60

The analysis of variance has found a significant effect for the sampling time for each coagu¹ ¹on parameter (p < 0 05) Cosinor analysis validated a circadian rhythm ᵦ for plasma fibrinogen (p<0 05).
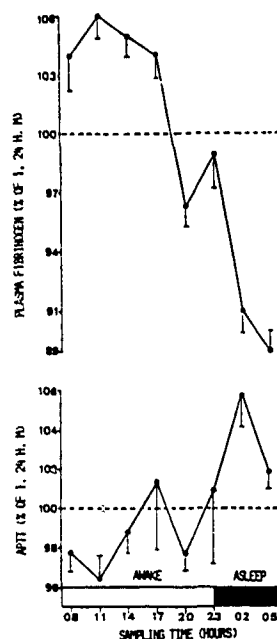
Figure 1   Time course of plasma fibrinogen and activated partial thromboplastin time expressed as percentages of the individual 24 hour mean (% of [24 H M), measured every 3 hours for 24 hours in eight healthy subjects. Vertical bars represent means (SEM) Significant circadian (24 hours) and ultradian (8 and 12 hours) rhythms were validated by cosinor analysis for these two parameters.

DISCUSSION

These results clearly found a circadian variation in coagulation processes (Figure 1). There was a morning hypercoagulability (peak for plasma fibrinogen and trough for APTT) and a nocturnal hypocoagulability (trough for plasma fibrinogen and peak for APTT). This circadian pattern was more prononced for plasma fibrinogen.

The circadian rhythms in plasma fibrinogen and acute vascular events seem to correlate directly (morning peak - nocturnal trouth) suggesting a cause-effect relation ship This fact reinforces the idea that plasm fibrinogen plays an important role in the genesis of these thrombotic disorders. It is not surprising since raised levels of fibrinogen may promote thrombogenesis by two general pathways (increase in platelet aggregability (8) and in blood viscosity (9))

The spontaneous circadian variation found in APTT is very close that observed for APTT measured ex vivo during heparin therapy (morning trough and nocturnal peak (6) and in vitro when heparin was added to human plasma sampled at different times of day (morning trough and nocturnal peak (10)) This suggests that heparin might amplify the spontaneous circadian rhythms in coagulation processes

Such data are consistent with those of other studies performed on circadian changes of hemostatic system in man. An other experiment (11) found a morning peak for plasma fibrinogen and a morning trough for thrombin time. In addition, the level of platelet aggregability has been found to be increased in the morning hours (12) whereas the fibrinolytic activity was lower at this time of the day (13).

Further circa' ~n evaluations of coagulation processes are needed in high risk patients. If these studies confirm our results pharmacological approach to decrease high levels of plasma fibrinogen, especially in the morning hours, would deserve to be undertaken.

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ACKNOWLEDGMENTS

INTRAPULMONARY ADMINISTRATION
OF HEPARIN IN THE MORNING AND
ITS EFFECT UPON BLOOD VARIABLES

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INTRODUCTION

Advances in medical treatment and healthier lifestyles are believed to be the major factors which contribute substantially to the declining death rates from heart attacks, stroke and other cardiovascular diseases. Among the medical modalities in the treatment of coronary artery disease, heparin appears to hold a greater potential than any other agent tested to neutralize the atherogenic progress at many of its stages (Engelberg, 1988). The self-administration of low level dose heparin by inhalation appears to be well justified and easily adapted to home therapy (Bick, 1985). The timing of inhalation may also be important in maximizing the desired effect and/or minimizing any undesired effect of intrapulmonary heparin. To test the feasibility of further clinical trials, a preliminary pilot study was performed on three volunteers in order to look at the response of variables in the blood to intrapulmonary heparin. Any blood variables which appear to be responsive to this route of delivery could serve as markers when low-dose heparin is administered.

SUBJECTS, MATERIALS AND METHODS

Three clinically symptom-free adults volunteered for this study: 2 men (EK, age 57; FB, age 47) and one woman (MR, age 47). Body size characteristics were: height: 1.73, 1.83, 1.63 m; weight: 79.4, 87.1, 49.9 kg; and body surface area: 1.92, 2.09, 1.52 m^2 for EK, FB and MR, respectively. Two weeks prior to and during the study, EK and MR abstained from taking aspirin. Subjects maintained their usual daily routines as hospital staff members. For one week before and during the entire study period, EK (a biochemist) maintained a low salt, low cholesterol diet, taking meals at 0700, 1200 and 1800. He also took medications at regular hours: Cardizem (60mg, t.i.d.), Capoten (25mg, b.i.d.), and Tenormin (25mg) and Mevacor (40mg) once a day. MR (a nurse) ate a balanced, low calorie diet, with meals at 0700, 1200 and 1800. FB (a cardiologist) ate irregularly, but selectively, and attended a banquet in the evening of the heparin study day.

Control Period (Monday, January 8, 1990): Four ml of distilled water was placed into a nebulizing chamber (Porta-Sonic Ultrasonic Nebulizer, Model 8500-D, producing particle sizes ranging from 1-3 microns [DeVilbiss Health Care Worldwide, P.O. Box 635, Somerset, PA., 15501-0635, USA]). Inhalation by slow, deep breaths began at 0745 and continued for exactly 10 minutes until 0755. Prior trials indicated that the volume of water mist inhaled would be between 1.0-1.5 ml. Pulmonary function measurements (FEV-1, using a Vanguard Digital Spirometer D.S. 601) and blood collections were made at the following clock hours: 0800, 0900, 1000, 1300, 1600, 1900, 2200, 0100, 0400, 0700 and 1000 (n=11). Blood was collected into 3 separate vacutainers: one without anticoagulant, one with citrate and one with EDTA. After each collection the samples were taken to the laboratory for assays of heparin, APTT, TT, fibrinogen and standard RBC and WBC hematology. The clotted blood was centrifuged, serum aliquoted and stored for chemical, lipid and hormonal assays.

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Heparin Period (Monday, January 15, 1990): Four ml of Sodium Heparin (10,000 U/ml, derived from porcine intestines) was placed into each nebulizing chamber. Inhalation began at 0745 and continued for 12 minutes for EK and FB and for 15 minutes for MR. The exact amount of heparin that was inhaled was calculated from the difference in weight of 4.0 ml (4.13 gm) heparin before inhalation and the remaining weight of heparin after inhalation. Actual units of heparin inhaled were calculated to be 12,000 (210 U/kbw), 14,700 (169 U/kbw) and 10,500 (151 U/Kbw) for EK, FB and MR, respectively. Pulmonary function measurement and blood samplings were made at exactly the same hours as during the control period. As follow-up, additional samples were obtained from EK and MR at 1000 on days 3, 4, 5, 6, 7, 8, 9 and 14 after heparin.

Circadian rhythm characteristics of Mesor, amplitude and acrophase were derived for each variable by subject during the control and heparin periods by the least-squares fit of a 24h cosine (Nelson, 1979). Rhythm characteristics were compared by the Bingham test of rhythm parameters (Bingham, 1982).

RESULTS

No heparin was detected in blood and no increases were noted in APTT or TT. Rhythm-adjusted 24h averages (Mesors) for each subject are presented in Table 1 for the control and heparin periods, as well as direction and magnitude (%) of difference and an indication of significance as determined by Bingham mesor test. Same direction changes in all 3 subjects were noted for Mesor (*increase* for basophils, platelets, Ca/Mg ratio and ANF1-98; *decrease* for TSH, lymphocytes, monocytes, eosinophils, cholesterol, insulin, prolactin, cortisol, Na, K, Mg and MPV) and amplitude (*increase* for TT, fibrinogen, Mg and P; *decrease* for RBC, monocytes, eosinophils, cholesterol, insulin, LDH, K and Ca). Although most of these changes were minimal and never exceeded the normal range, parameter testing resulted in statistically significant changes at p≤0.05 in all 3 subjects as follows: Mesor *increases* in platelets (+12%) and Ca/Mg ratio (+15%) and *decreases* in TSH (-29%), Na (-2%) and Mg (-14%). Some circadian waveforms of interest are presented in Figs 1-4 for platelets, cholesterol, TSH and ANF99-126, respectively.
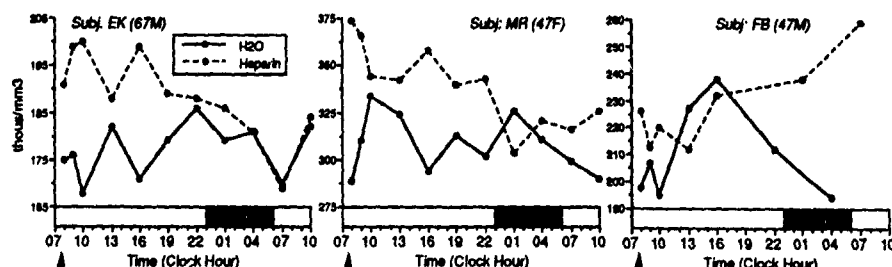

Fig 1: Platelets for 24 Hours Following H2O or Heparin Inhalation in the Morning (arrow)
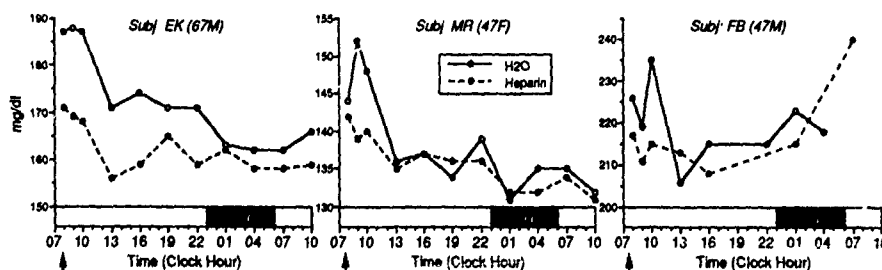

Fig 2: Serum Cholesterol for 24 Hours Following H2O or Heparin Inhalation in the Morning (arrow)

Table 1: Comparison of Circadian Mesors in Blood Variables and FEV
following Inhalation of H20 (Day 1) or Heparin (Day 2) at 0800*

| Subj: | | EK | | MR | | FB | | Day 1 to 2 change (%) | | | |
Variable	units	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	EK	MR	FB	Mean
FEV		87.6	85.8	91.1	90.3	-	88.2	-2.1	-0.9		-1.5
Hep Test	secs	19.20 ** 16.82		20.99	20.60	16.67	16.80	-12.4	-1.9	0.8	-4.5
APTT	"	26.16	25.64	27.76	28.09	26.96	26.66	-2.0	1.2	-1.1	-0.6
TT	"	14.41	14.38	14.44	14.56	14.78	14.28	-0.2	0.8	-3.4	-0.9
Platelets	thou/mm3	178 *** 189		309 *** 338		212 * 233		6.2	9.4	9.9	8.5
MPV	fl	11.27 * 11.05		9.42 ** 9.20		10.85 * 10.49		-2.0	-2.3	-3 3	-2.5
Fibrinogen	mg/dl	382 * 434		204	188	324	317	13.6	-7.8	-2.2	1.2
Triglycerides	"	112 *** 99		89	85	297	395	-11.6	-4.5	33.0	5.6
Cholesterol	"	171.6 ** 161.7		137.4	135.5	218.2	215.6	-5.8	-1.4	-1.2	-2.8
HDL	"	52.2	50.6	64.2	62.6	36.6	35.8	-3.1	-2.5	-2.2	-2.6
LDL	"	101.2 ** 95.2		59.1	59.6	134.4 ** 116.7		-5.9	0.8	-13.2	-6.1
VLDL	"	18.1 *** 15.8		14.0	13.4	47.4	63.2	-12.7	-4.3	33.3	5.4
RBC	mil/mm3	4.61	4.56	3.95 *** 3.69		4.55	4.52	-1.1	-6.6	-0.7	-2.3
WBC	cells/mm3	6318	5937	7876 *** 6176		6334	6756	-6.0	-21.6	6.7	-7.0
Neut	%	65.7	65.2	58.8 *** 52.2		44.4	48.7	-0.8	-11.2	9.7	-0.8
Lymph	"	25.7	26.7	34.5 *** 40.1		42.6 *** 39.1		3.9	16.2	-8.2	4.0
Mono	"	5.5	5.4	5.0 . 5.6		7.2	6.6	-1.8	12.0	-8.3	0.6
Eos	"	2.7 *** 2.1		1.3	1.5	4.6	4.0	-22.2	15 4	-13.0	-6.6
Baso	"	0.46	0.59	0.52	0.69	1.22	1.64	28.3	32.7	34.4	31.8
Neut	cells/mm3	4157	3878	4598 *** 3190		2816 * 3280		-6.7	-30.6	16.5	-7.0
Lymph	"	1618	1578	2743	2506	2697	2666	-2.5	-8.6	-1.1	-4.1
Mono	"	347	321	392	343	454	439	-7.5	-12.5	-3.3	-7.8
Eos	"	169 *** 123		102	97	291	265	-27.2	-4.9	-8.9	-13.7
Baso	"	29	35	40	43	77	108	20.7	7.5	40.3	22.8
Gastrin	pg/ml	680	769	115 ** 101		62	98	13 1	-12.2	58.1	19.7
PTH	"	46.0 ** 52.8		32.1	32.1	qns	qns	14.8	0.0	qns	7.4
Insulin	μU/ml	20.0	16.1	19.0	18.3	20.6	23.6	-19.5	-3.9	14.6	-2.9
Renin	ng/ml/hr	1.03 ** 0.58		1.89 *** 0.93		0.88 * 1.45		-43.7	-50.8	64.8	-9.9
TSH	μIU/ml	4.40 *** 3.47		1.11 ** 0.82		2.28	1.39	-21.1	-26.1	-39.0	-28.8
Prolactin	ng/ml	10.03	9.39	11.66	10.80	10.01	8.00	-6 4	-7.4	-20.1	-11.3
Cortisol	μg/dl	9.52	8.49	6.96	6.95	8.48 * 5.18		-10.8	-0.1	-38.9	-16.6
SGOT	U/L	24.30 *** 27.10		23.47 * 22.35		28.21 ** 24.31		11.5	-4.8	-13.8	-2.4
SGPT	"	13.50	13.80	5.50	4.56	20.24	20.22	2.2	-17.1	-0.1	-5.0
LDH	"	162.9 *** 174.9		122.3	134.3	191.5	165.9	7.4	9.8	-13.4	1.3
GGT	"	32.10 *** 27.80		8.47 * 7.57		31.53	33.43	-13.4	-10.6	6.0	-6.0
ANF1-98	pg/ml	1950	1954	2017	2069	1765	1780	0.2	2 6	0.8	1.2
ANF99-126	"	70.4	70.3	71.4	70.4	63.5 ** 78.6		-0.1	-1.5	23.8	7.4
Na	mEq/L	144.2 ** 142.0		141.0	140.2	143.3 *** 138.7		-1.5	-0.6	-3.2	-1.8
K	"	4.67 *** 4.12		4.6	4.46	5.36	4.96	-11.8	-3.0	-7.5	-7.4
Ca	mg/dl	10.1 *** 9.83		9.57	9.58	9.79	9.69	-3.1	0.1	-1.0	-1.3
Mg	"	2.14 *** 1.86		2.16 *** 1.90		2.35 *** 1.98		-13.1	-12.0	-15.7	-13.6
P	"	3.48 * 3.23		3.86 *** 4.28		3.10 *** 3.60		-7.2	10.9	16 1	6.6
Ca/Mg	ratio	4.75 *** 5.29		4.44 *** 5.06		4.17 *** 4.90		11.4	14.0	17.5	14.3

*Significant difference from Bingham parameter test indicated as follows: *** p≤0.01, ** p≤0.05, * p≤0.10

DISCUSSION

In this pilot study limited to only 3 adults, inhalation of low-dose heparin did not cause any discomfort or reveal signs of any allergic reaction, while pulmonary function remained unaffected and the many blood variables examined were minimally or not affected at all. Meaningful assessment of acrophases will have to wait until the study is expanded to larger numbers of subjects and dosing at different circadian stages. In focusing on the 24h mean value before and after heparin administration, we chose to compare 24h rhythm-adjusted means (Mesors), rather than arithmetic means, since the latter were biased by the unequidistant sampling schedule resulting in increased sampling in the morning hours. Even though the current study is limited to only 3 subjects, it was important to note that all values observed after heparin inhalation were within normal ranges, indicating the relative safety of this route of administration.
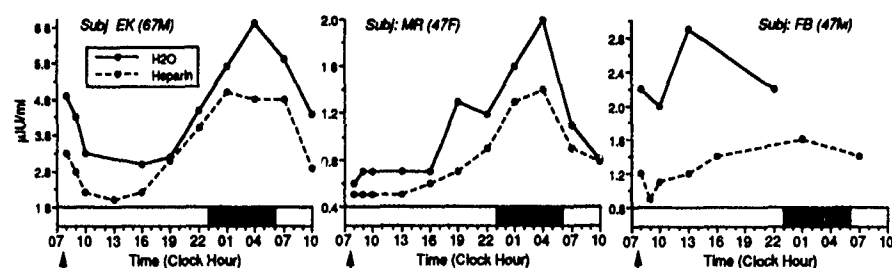
Fig 3: Serum TSH for 24 Hours Following H2O or Heparin Inhalation In the Morning (arrow)

What appears to be needed at this time is the standardization of low-dose heparin administration with respect to time and the identification of some markers in the blood which would accurately and conveniently monitor its effect. The significance of proper timing in blood sampling for heparin, APTT, TT and coagulation factor X has been demonstrated by Decousus and colleagues (1985). In this study, heparin was administered intravenously to 6 patients by a constant infusion pump. The results of coagulation tests revealed circadian variation in all patients. Maximum values were achieved at around midnight and minimum values were noted in the morning hours.

Speculations on analytes investigated in the present study as affected by the low-dose heparin, and their usefulness as markers in heparin therapy, must await confirmatory trials in a larger number of subjects. These trials should also include other circadian schedules in its administration and sampling. The presently gathered data strongly suggest that circadian timing of sampling of preselected variables must be synchronized with the timing of heparin administration. Such standardization is essential to the accurate monitoring and interpretation of heparin effects.
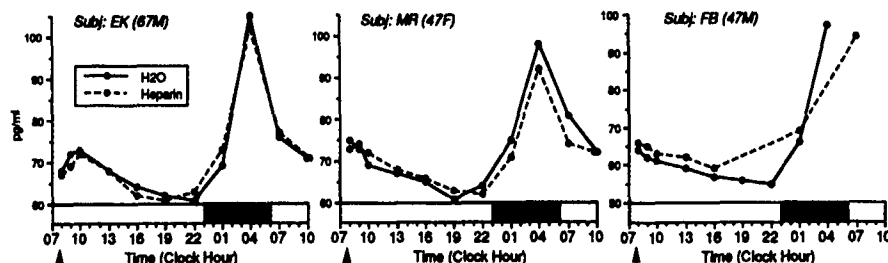
Fig 4: Serum ANF99-126 for 24 Hours Following H2O or Heparin Inhalation In the Morning (arrow)

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CIRCADIAN TIMING OF RECOMBINANT HUMAN ERYTHROPOIETIN (rhEPO) IN THE MOUSE AFFECTS HEMATOPOIETIC RESPONSE

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INTRODUCTION

Many hormones are released in characteristic non-random circadian patterns. In addition, the responsiveness of specific target tissues often vary reproducibly throughout the day. For example, adrenocorticotropin (ACTH) is released in frequent spikes which are patterned on the circadian time scale. The adrenal target cells also respond predictably better at circadian stages associated with daily ACTH maxima (Ungar and Halberg, 1962). Erythropoiesis is known to be highly rhythmic on the circadian (24 hour) time scale with respect to serum levels of erythropoietin (Wide et al., 1989), erythroid progenitor cell numbers, the resultant number of reticulocytes and mature erythrocytes (Haus et al., 1983), and in the susceptibility to myelotoxic drugs (Laerum et al., 1989). The biologic and biochemical basis for the coordination of these endogenous hematopoietic rhythms remains to be explained. The circadian variation in serum glucocorticoid levels in man and animals is well known. These steroids, at physiologic concentrations, have been shown to promote the survival and proliferation of human erythroid and myeloid precursors *in vitro* (Chikkappa and Pasquale, 1989; Chikkappa and Pasquale, unpubl.). The role of these steroids in coordinating the circadian-based hematopoietic rhythms has not been defined. The time structure of other stimulatory (interleukin (IL)-3, IL-1, GM-CSF) or inhibitory (ferritins) factors affecting erythropoiesis have not be described.

Recombinant human growth factors and cytokines are now readily available with many potential clinical applications. However, our knowledge of how to administer these proteins is suboptimal. The *in vivo* efficacy of some of these proteins has been less than theoretically predicted and some proteins have an extremely narrow therapeutic (efficacy/toxicity) ratio. There is some evidence that the circadian timing of administration of some of these proteins (e.g. tumor necrosis factor) can reduce

toxicity *in vivo* without compromising certain desired biologic effects (e.g. antitumor activity) (Langevin et al., 1987). Therefore, endogenous circadian biologic variations might be exploited to more safely, inexpensively and effectively deliver these expensive and often toxic cytokines. As a first step toward better understanding the mechanisms of the circadian coordination of erythropoiesis, we investigated the circadian time dependence of reticulocyte and hematocrit response to rhEPO (recombinant human erythropoietin) in the mouse.

METHODS AND RESULTS

Ninety CD_2F_1 female mice, 11 weeks old, were adapted to an alternating schedule of 12 hours of lights on and 12 hours of lights off. Circadian synchronization was documented by the characteristic daily rhythm in rectal temperatures. Mice were injected subcutaneously with a single dose of 300 Units/kg of rhEPO (Ortho Biotech) at one of six different circadian times. Erythropoietic response was evaluated by hematocrit and reticulocyte determinations on heparinized blood obtained by exsanguination of animals at 24 hour intervals over five days following injection of rhEPO. Reticulocyte percentages were determined by new methylene blue staining of blood smears. Time is referenced relative to the Hours After Lights On (HALO) in these nocturnally active animals.

As previously described, baseline hematocrit and reticulocyte percentages each varied as a function of circadian stage of measurement. The response in hematocrit and reticulocytes to rhEPO in CD_2F_1 mice varied with the circadian time of injection of the hormone. The increase in reticulocytes was usually maximum at 48 hours. For a fixed dose of rhEPO, the rise in reticulocytes varied from a 50% increase when injected at 23 or 1 HALO (early light, beginning of rest in nocturnal animals) to a 310% increment when injected at 15 HALO (early dark, beginning of activity) (Fig. 1).
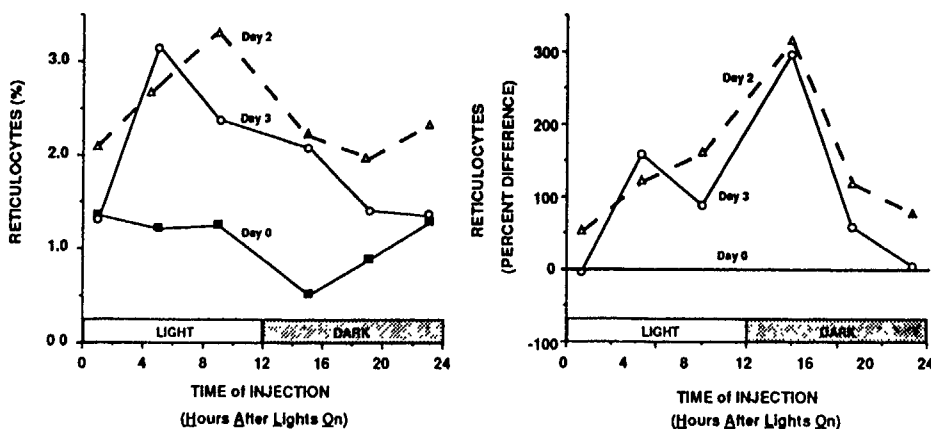
Fig. 1. Circadian dependent reticulocyte response to erythropoietin (rhEPO) in CD2F1 female mice. Reticulocyte percentages were determined on blood smears at 24-48 hour intervals on groups of mice injected subcutaneously with a single dose of 300U/kg rhEPO at one of six circadian stages referenced to hours after lights on (HALO). Mean±SEM are shown. Values are expressed as absolute percentage of cells determined to be reticulocytes (left figure) and as the percent change (right figure) from the same circadian time prior to treatment (day 0). Days 2 and 3 are shown, being the time of maximal overall response.

Over the 5 days following injection of rhEPO, the hematocrit response varied from a 9% increase at 72 hours when given at 23 or 1 HALO to a lack of a significant elevation when given at four other intermediate HALO's (Fig. 2). Erythropoietin injection also resulted in an early (24-48 hours) and previously unreported decrease in hematocrit. The magnitude and timing of this rhEPO-associated initial fall in hematocrit was also circadian stage dependent.
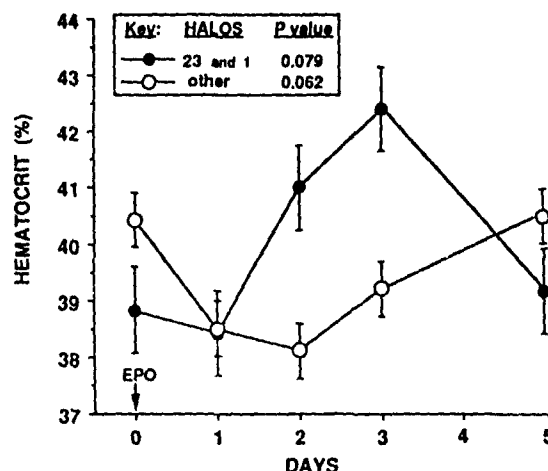
Fig. 2. Circadian dependent hematocrit response to erythropoietin (rhEPO) in CD2F1 female mice. Hematocrits were determined at 24-48 hour intervals on groups of mice injected subcutaneously with a single dose of 300U/kg rhEPO at one of six circadian stages. Data for injections at 23 and 1 HALO (hours after lights on) are pooled and that from the other four intermediate HALO's are pooled and the means±SEM are shown with p values from one way ANOVA.

DISCUSSION

The biologic effects of erythropoietin vary markedly as a function of when during the circadian cycle, the same amount of hormone is administered to mice with normal hematocrits. The reticulocyte response to rhEPO varies almost 7-fold as a function of its circadian timing with the greatest response occurring when rhEPO is given shortly after usual awakening as compared to later in the day. This maximal reticulocyte response to rhEPO is coincident with the predicted peak in serum corticosterone levels in mice. In man, the percentage of nucleated cells in the bone marrow in S (synthetic) phase sampled throughout a 24 hour period does correlate positively with serum cortisol levels with a phase delay in the former of about 4 to 8 hours (Smaaland et al., 1990). These potential interactions of corticosteroids, erythropoietin and eythroid precursors are readily testable both in vitro and in vivo and are the basis for future investigations.

A circadian stage dependent early decrement followed by a later increase in hematocrit was observed with rhEPO administration. The mechanism of this unexpected decrease in hematocrit remains unexplained. The time course for the early decrease in hematocrit would appear to be too rapid to be explained by hematopoietic effects. Hormone- or vehicle-induced rapid changes in splenic seques.ration of erythrocytes or changes in plasma volume might be more likely explanations. Further delineation of the mechanisms involved and the clinical relevance of this paradoxical effect of rhEPO are needed.

Careful and quantitative observation of the circadian time structure of erythropoiesis may aid in the understanding and delineation of the complex variables relevant to the *in vivo* hematopoietic cascade. Optimal growth factor timing offers a simple and effective strategy to maximize desired biologic effects of erythropoietin with the possibility of decreasing frequency or amount of drug, thereby decreasing the cost and inconvenience of effective therapy. Subsets of patients in clinical rhEPO trials for various anemias have either not responded, responded very slowly, or required high doses of rhEPO. When nutritional deficiencies (e.g., iron) or toxicities (e.g., aluminum) have been excluded, perhaps some of these so called 'erythropoietin refractory anemic states' may be more effectively treated by optimum circadian timing of rhEPO.

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TEMPORAL VARIATION IN ACUTE NEUROTOXICITY OF TRICHLOROETHYLENE IN THE RAT

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ABSTRACT

Temporal variation in acute neurotoxicity of trichloroethylene (TRI:1.2g/kg
i.p.) was investigated in the rat under a light:dark=12:12 hr cycle. Neurotox-
icity evaluated by the decreased muscle tone was maximal at an early dark phase
(21.00) when the metabolic elimination of TRI in the liver was the lowest.
Neurotoxicity was also evaluated by a continuous recording of spontaneous
locomotor activity in the rat. Circadian rhythm in spontaneous locomotor
activity was considerably impaired by the injection of TRI for three consecu-
tive days. Spectral analysis showed that ultradian components became more
dominant than circadian component. The degree of circadian rhythm impairment
seemed greater when injected at 09.00 than 21.00.

KEY WORDS

Trichloroethylene; neurotoxicity; circadian rhythm; spontaneous locomotor
activity; power spectrum.

INTRODUCTION

Trichloroethylene (TRI) is widely used as a degreasing agent in industry and
toxicological studies of it have become an urgent problem (Kimbrough et al.,
1985). The major toxic effects of TRI are depression of the central nervous
system and liver damage (Reynolds et al., 1979). We have previously reported
the chronohepatotoxicity of acute administration of TRI in a LD=12:12 hr cycle
and its alteration in a free running condition (Motohashi et al., 19℃). In
this study, we investigated temporal variations in the acute neurotoxicity of
TRI in the rat by assessing decreased muscle tone and measuring spontaneous
locomotor activity.

MATERIALS AND METHODS

Male albino Wistar-strain rats aged 6 weeks were housed in a LD=12:12 hr
cycle (light on 08.00 to 20.00) with food and water ad libitum. Room temperat-
ure and relative humidity were maintained regulated at 25+0.5°C and 60+5%,
respectively. Two experiments were carried out. (Experiment 1) Forty-four
rats were divided into a TRI-injected groups and control groups. Intraperito-
neal injection of TRI (1.2 g/kg body weight) or saline were conducted simulta-
neously (six rats in each TRI group and five in each control group) at 03.00,
09.00, 15.00, and 21.00, respectively. Thirty minutes after injection, an
"inclined plane test" was carried out to assess the acute neurotoxic effect

(decreased muscle tone) of TRI. A smooth surface plane was inclined at an
angle of 30° and a rat was placed on the top of this inclined plane. The
number of rats that slipped and fell was scored. Furthermore, the two hour
after injection, rats were anesthetized by ethylether and venous blood were
sampled. Trichloroethanol (TCE) and trichloroacetic acid (TCA) concentrations
were determined by gas-chromatography with ECD detector.

(Experiment 2) Spontaneous locomotor activity of rat was continuously moni-
tored by the animal activity recorder (Animex IIIA, Shimazu) in order to inves-
tigate subacute effects of TRI injection. Animex IIIA monitored the animal
activity by detecting changes in capacitance due to the animal activity. The
experiment was carried out for 20 to 30 days with a sampling interval of 15
minutes. The effects of intraperitoneal injection of TRI on circadian rhythm
in the spontaneous locomotor activity was investigated. The injection of TRI
(1.2 g/kg/day) was conducted for three consecutive days at 09.00 (light phase)
or at 21.00 (dark phase). Time series data were analyzed by spectral analysis
with maximal entropy method, 1/f fluctuation analysis and cosinor analysis.
The 1/f fluctuation is defined as the fluctuation whose power spectral density
is inversely proportional to frequency (Voss et al., 1975; Kobayashi et al.,
1982). In this report, the 1/f fluctuation is used for the evaluation of
neurotoxicity.

     RESULTS

The temporal variation in neurotoxic effect of TRI was observed by an inclined
plane test (Table 1). The rates of rats slipped and fallen were 50% (03.00),
33% (09.00), 7% (15.00) and 67% (21.00), respectively. Thus, the peak time of
neurotoxic effect of TRI was an early active phase (21.00). For the evaluation
of elimination of TRI by the drug metabolism in the liver, temporal variations
in metabolites of TRI were investigated. The peak concentrations of TCE and
TCA were $35.2 \pm 8.8 \mu mol/l$ and $170.0 \pm 4.1 \mu mol/l$, respectively, when injected at
09.00.

Spontaneous locomotor activity of the rats was greatly decreased by treatment
with TRI on the days of injection. After the injection of TRI, the circadian
rhythm of spontaneous locomotor activity was considerably impaired. Fig. 1.
represents a double plot of the activity rhythm a rat injected at 09.00 and at
21.00. The circadian rhythm of spontaneous locomotor activity was not restored
until 8 days after the end of injection of TRI when injected at 09.00, while
the duration of circadian rhythm impairment was three days when injected at
21.00. The 1/f fluctuation observed before injection of TRI disappeared after
the injection of TRI. Spectral analysis showed that ultradian components
(τ=6h, 12h) became more dominant than the circadian component after the injec-
tion of TRI. Circadian rhythm impairment caused by TRI seemed greater when
injected at 09.00 than at 21.00.

Table 1 Results of the inclined plane test and the concentrations
         of trichloroethanol (TCE) and trichloroacetic acid (TCA) in
         the blood after the injection of TRI in a LD=12:12 hr cycle.

Time of day	Inclined plane test*	Trichloroethanol (TCE) concentration ($\mu mol/l$)	Trichloroacetic acid (TCA) concentration ($\mu mol/l$)
03.00	50 %	22.9 ± 6.5**	161.1 ± 13.7
09.00	33 %	35.2 ± 8.8	170.0 ± 4.1
15.00	7 %	21.9 ± 7.7	147.5 ± 14.1
21.00	67 %	20.6 ± 5.2	153.3 ± 21.2

     * The rates of rats slipped and fallen
     ** Mean $\pm$ standard deviation is shown
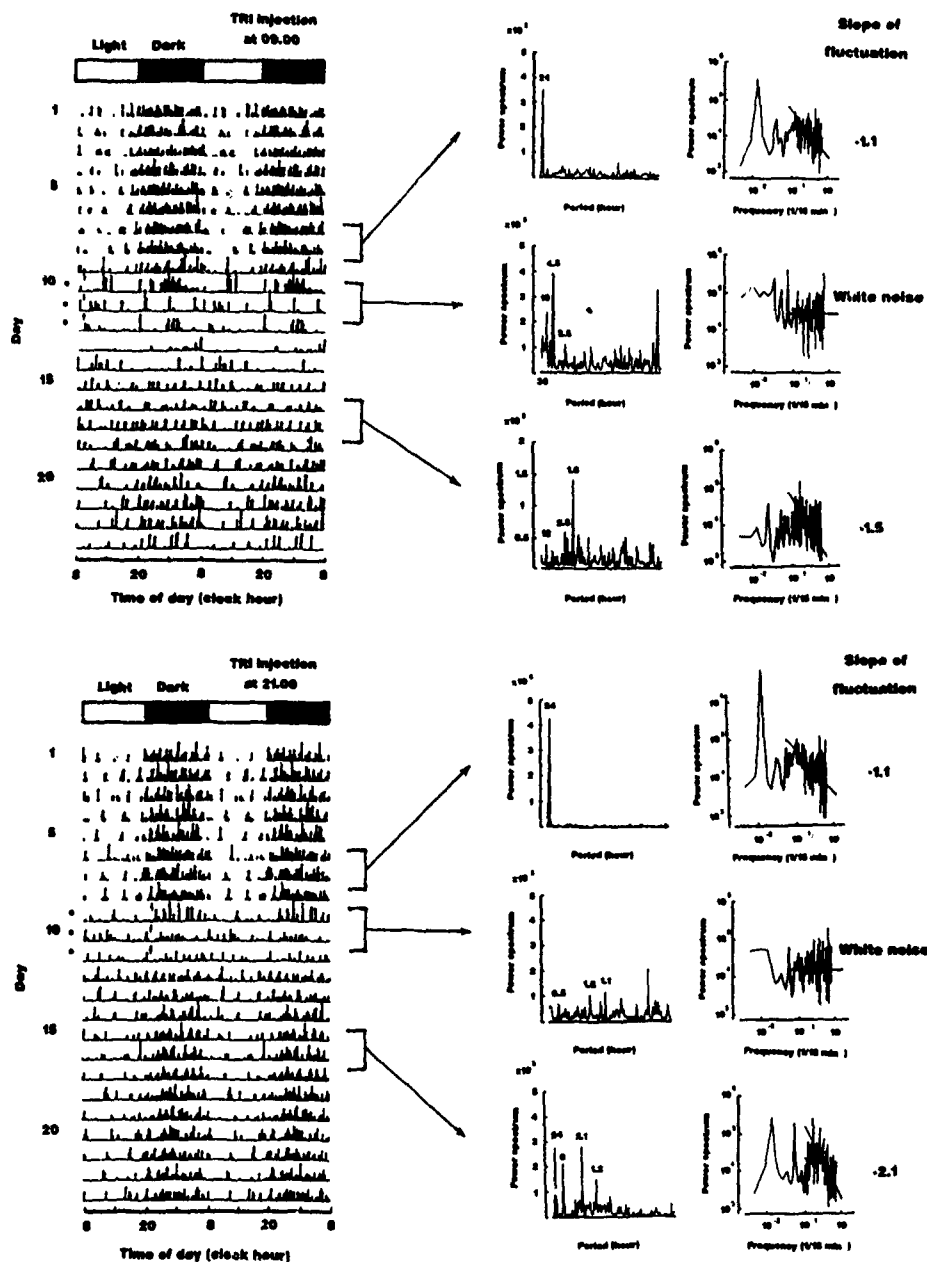
Figure 1. The effects of trichloroethylene (TRI: 1.2g/kg, i.p) on spontaneous locomotor activity in LD=12:12 hr are shown. The data are double-plotted to facilitate visual inspection of the records. Injection times were 09.00 (upper) or 21.00 (lower). The days and times of TRI injection were indicated by closed circle (●) and arrow (↓), respectively. Power spectrum with maximal entropy method and 1/f fluctuation analysis are also shown. The circadian rhythm was considerably impaired by injection of TRI at both 09.00 and 21.00. The degree of impairment was greater when injected at 09.00. The 1/f fluctuation disappeared after the injection of TRI at both 09.00 and 21.00.

DISCUSSION

The present study revealed that the acute neurotoxicity of TRI evaluated on the basis of decreased muscle tone was maximal at 21.00 corresponding to an early active phase in the rat. Such a circadian variation in depressive effect on the central nervous system of a lipophilic chemi ʌl agent was reported in the case of many anesthetics (Bruguerolle, 1984; Bruguerolle, 1988), although the time of peak toxicity differs from substance to substance. In this study, the blood concentrations of TRI metabolites, TCE and TCA, were determined to assess metabolic elimination of TRI in the liver. Since the concentration of TCE was maximal at 09.00 and minimal at 21.00, the acute neurotox·city of TRI was accentuated during the phase when metabolic elimination by the liver was lowest. This suggests that the higher the TRI concentration in the blood, the greater the neurotoxicity of TRI. It is noteworthy that the circadian peak phase of acute of neurotoxicity of TRI was a trough phase of hepatotoxicity as reported previously (Motohashi et al., 1990), in which TCE concentration in the blood played an important role. In contrast, the circadian rhythm in spontaneous locomotor activity was more impaired by the injection of TRI at 09.00 than at 21.00. In other words, the treatment with TRI during the early resting phase provoked greater circadian rhythm impairment as a subacute effect. It was possible to quantitatively evaluate impairment circadian rhythm by TRI on the basis spectral analysis and 1/f fluctuation analysis. Such temporal variations of acute and subacute effects of TRI should be taken into consideration in industrial toxicology, although the reason of their mechanisms should be the subject of further investigation.

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A HISTOCHEMICAL DEMONSTRATION BY DIGITAL IMAGING MICROSCOPY OF THE VALUE OF THE CIRCADIAN RHYTHM OF ALKALINE PHOSPHATASE ACTIVITY IN RENAL CHRONOTOXICOLOGY

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ABSTRACT

In investigating the mechanisms involved in renal chronotoxicology, it has been speculated that brush border enzymes may play a key-role (Cal et al., 1989). In this study, the in situ activity of alkaline phosphatase (AlP) was examined in Sprague-Dawley rats of both sexes maintained on a 12-hr LD cycle (light on at 0800 hr) injected with a single i.p. dose of 1 mg Hg/kg of mercuric chloride or of saline (control groups) at one of four circadian stages: 0500 hr, 1100 hr, 1700 hr, or 2300 hr (in June). AlP activity was estimated by digital imaging microscopy in cryostat sections of kidneys removed 24-hr after dosing, after a treatment based on the Calcium-Cobalt method of Gomori (1946). Statistically significant circadian variations of AlP were observed in saline-treated rats of both sexes with a peak at the end of the rest period. In rats injected with HgCl2, a circadian rhythm of AlP was also demonstrated but with a peak occurring in the middle (female) or at the end (male) of the dark span indicating a higher resistance to the nephrotoxicant at this time. The findings suggest that the higher activity of renal AlP at the end of the light span results in a greater susceptibility to functional loss and cellular necrosis associated with mercuric chloride nephrotoxicity. In addition, the timing and the amplitude of the circadian rhythm of renal AlP appeared to be influenced by sex.

KEY WORDS

Alkaline phosphatase, image analysis, chrononephrotoxicity, histochemistry, mercuric chloride.

INTRODUCTION

Time-dependent rhythmic variations of renal susceptibility to nephrotoxic drugs as shown especially by urine concentrating nephron ability or by enzymuria have been increasingly reported for the last decade (review in Cal et al., 1989). However, the mechanisms underlying these variations cannot yet be explained. It seems nevertheless reasonable to suggest that the many functions of the kidney which are known to exhibit diurnal variations e.g. glomerular filtration rate (Cambar et al., 1979), renal blood flow, electrolyte excretion (Wesson, 1964) or enzyme urinary activity (Cal et al., 1987) may have a relatively specific role. Similarly, the role of several hormones and particularly of sexual hormones is believed to be of importance in the timing of peak enzyme activity as well as in the resistance of the kidney to nephrotoxic injury (Cal et al., 1989). Against this background, we have set out to verify and extend previous findings (Cal and Cambar, 1984) on the circadian rhythm of tubular injury induced by mercuric chloride (as one of the best models of acute renal failure). To this purpose, the epithelial activity of alkaline phosphatase (E.C. 3.1.3.1., AlP) a brush border enzyme, which is inhibited by the heavy metal during the first stages of tubular necrosis (Zalme et al., 1976), was measured by quantitative histochemistry at various times of day, in control and intoxicated rats of both sexes. This goal was achieved by using for the first time, to our knowledge, digital imaging microscopy in this kind of application.

MATERIALS AND METHODS

Animals and sampling. 24 male and 24 female Sprague-Dawley rats were maintained for two weeks under constant environmental conditions in a temperature ($22 \pm 1°C$) and humidity ($50\% \pm 5\%$) - controlled room. They were allowed free access to food and water and exposed to a 12-hr light/dark cycle (0800/2000). Male (250 ± 10 g) and female (200 ± 10 g) eight-weeks old animals received a single i.p. injection of 1 mg/kg of mercury as mercuric chloride or of saline (control groups) at one of the four following equispaced circadian stages : 0500 hr, 1100 hr, 1700 hr or 2300 hr (in June). 24 hr after dosing, the animals were killed by an excess of pentobarbital and the right kidney removed immediately, frozen in liquid nitrogen and stored at - 80°C.

Histochemistry. 6 µm cryostat sections were cut at a cabinet temperature of - 25°C and picked up onto glass slides. The incubation for AlP was based on the Calcium-Cobalt method of Gomori (1946). Slides were placed for 15 min at 37°C in a mixture made up of 2% sodium barbital and 2% sodium-ß-glycerophosphate (as the substrate) 10 ml each, 2% $CaCl_2$ 2 ml, 2% $MgSO_4$ 1 ml and distilled water, 20 ml. After washing, the slides were immersed in a solution of cobalt nitrate (2%) for 2 min and, after rinsing, in 2% $(NH_4)_2S$ for 2 min. Sections were mounted in polyvinylpyrolidone after rinsing. Control incubations were performed in the absence of substrate. Areas of AlP action stained brown - black due to the conversion of the precipitated calcium phosphate into cobalt sulphide.

Quantitative Histochemical Analysis. Slides were analysed by digital imaging microscopy with a Biocom system consisting of a Compaq® microcomputer (640 Kb of RAM) and of the specific Biocom® software, Imal™ for image analysis coupled with a Leitz® microscope and a x10 objective. The specific optical density of the AlP spots was determined on a scale of 256 grey levels. A set of macroinstructions was written to allow an automatic calculation of the area of AlP spots. For each field observed, this area was recorded as a percentage of the total area of the field. At least five fields at the corticomedullary junction per slide were examined.

Statistical Analysis. All data are reported as mean ± standard error. Multiway analysis of variance was used when applicable for testing the effect of $HgCl_2$, time and sex on AlP epithelial activity. The overall variability of the data was assessed by the single cosinor method using a program specifically designed by J.C. Cal for the Macintosh® microcomputer.
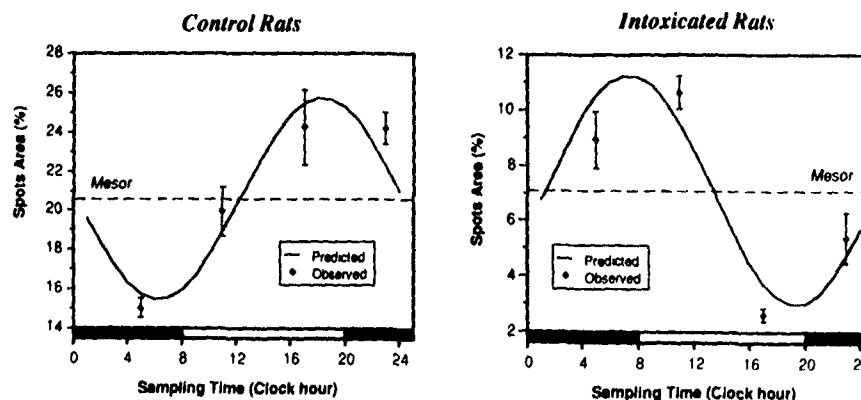

Fig. 1. Circadian variations of AlP activity in male rats, 24-hr after saline (left side) or after $HgCl_2$ (right side) injection, as assessed by the area of deposits as a percentage of the total area of the observed field: chronogram and best fitting cosine curve. The striped bars depict the dark period.

Single Cosinor

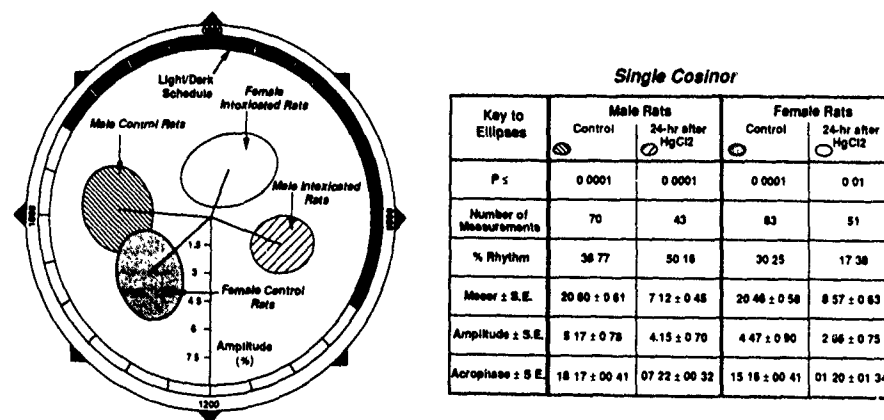| Key to Ellipses	Male Rats		Female Rats	
	Control	24-hr after HgCl2	Control	24-hr after HgCl2
P ≤	0 0001	0 0001	0 0001	0 01
Number of Measurements	70	43	83	51
% Rhythm	38 77	50 16	30 25	17 38
Mesor ± S.E.	20 60 ± 0 61	7 12 ± 0 48	20 46 ± 0 58	8 57 ± 0 63
Amplitude ± S.E.	8 17 ± 0 78	4.15 ± 0 70	4 47 ± 0 90	2 06 ± 0 73
Acrophase ± S E.	18 17 ± 00 41	07 22 ± 00 32	15 18 ± 00 41	01 20 ± 01 34

Fig. 2. Polar representation of the timing and phase relations of the circadian rhythms of AlP activity in male and female rats, 24-hr after saline or HgCl2 injection. Mesors and amplitudes are given in actual units i.e. spots area as a percentage of the total area of the field (time reference: local midnight).

RESULTS

Histochemistry coupled with digital imaging microscopy appeared as a powerful quantitative and non-subjective tool for the study of *in situ* renal enzyme activity. It made it possible to highlight significant circadian variations in renal AlP activity especially in control animals (p<0.0001), the area of AlP spots ranging in male rats from 15.03±0.52% at 0500 hr to 24.22 ±1.92% at 1700 hr (fig.1) and in female rats from 17.14±0.85% at 0500 hr to 25.32±1.04% at 1700 hr. A circadian rhythm was also shown in renal AlP activity 24 hr after mercuric chloride but the trough of these variations occurred at the timepoint of the maximum physiological AlP activity i.e. at the end of the light period for male rats (fig.1) and in the middle of the rest period for female rats (fig.2). Sex-related differences between the mesors of the AlP rhythms in control animals of both sexes were not observed. In contrast, a statistically significant fall of these mesors, 24-hr after HgCl2, was demonstrated by multiway analysis of variance (fig.2 - p<0.0001). Likewise, the influence of the circadian stage on AlP activity was statistically different in both sexes (p<0.005) and the administration of mercuric chloride caused statistically different shifts of the acrophase of AlP activity rhythms in male (11 hr) and female rats (14 hr - p<0.0001).

DISCUSSION

Renal alkaline phosphatases are non-specific alkaline phosphomonoesterases located at the brush border of the proximal tubules. As they are rapidly released in the urine due to their inhibition by nephrotoxic drugs (Zalme *et al.*, 1976), they are considered as early indicators of renal failure. The results of this study confirm this statement as the mesor of AlP rhythm fell 2.4 times in female rats and 2.9 times in male rats, 24-hr after HgCl2. They are also consistent with previous findings on the chrononephrotoxicity of mercuric chloride as assessed by enzymuria (Cal and Cambar, 1984) since it was demonstrated a circadian rhythm of AlP excretion with a peak at the end of the light span. This study provides thus additional evidence of the value of renal AlP as a marker for the chronosusceptibility of the kidney to nephrotoxicants.

Furthermore, in considering the many factors which might be involved in the genesis of the nephrotoxicity rhythms, the critical event leading to heavy metals-induced proximal tubular necrosis is considered to be their binding to sulfhydryl groups of tubular cells (Berndt *et al.*, 1985). Consequently, cellular enzymes which are, among other proteins, rich in thiols are primarily involved in the susceptibility of renal cells to metals. The present findings demonstrate that the crest of the physiological activity of AlP which occurs at the end of the light span in male rats for example, coincides with the time of the greatest toxicity of $HgCl_2$. Enzyme activities might thus be responsible for the 'phenotypic state' of tubular cells with regard to their chronotolerance to nephrotoxic drugs.

The reasons for the circadian variations of AlP in the healthy kidney remain to be ascertained but several hypotheses may be put forward. First, they could derive from the physics of the enzyme itself since spontaneous long period (up to 70 hr) oscillations of the catalytic capacity of malate dehydrogenase have been shown in solution under constant conditions (Queiroz-Claret *et al.*, 1988). Second, they could be due to bioperiodic changes in net synthesis or degradation of the enzymic protein. Third, they could be under the control of various hormones affecting their levels through their metabolism. The latter is of peculiar interest since it has been shown that AlP activity was dependent on the menstrual cycle in females (von Deimling, 1970) and that a marked loss of AlP activity occurred in female rats after castration (Albano and Rogonese, 1959). In addition, the present study suggests that the hormonal regulation or even sex-related differences influence the timing as well as the amplitude of the diurnal rhythm of AlP, 24-hr after $HgCl_2$. Indeed, the nadir of $HgCl_2$-toxicity occurred at the mid-dark span in females and at the end of the dark span in males whilst the amplitude of these rhythms represented 31% and 58.3% of the mesor respectively. Therefore, the results of this study support the assumption that sexual hormones may play a prominent role in the regulation and even the genesis of renal AlP activity rhythm.

It seems, thus, reasonable to suggest that the physiological rhythm of AlP might serve as a tool for predicting the susceptibility of the kidney to nephrotoxic drugs as it has been proposed that avian esterases serve as indicators of exposure to insecticides since they are also inhibited by the latter during the time course of birds intoxication (Thompson *et al.*, 1988).

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AGE-DEPENDENCY IN THE 24-HOUR-RHYTHMS OF BASAL AND FORSKOLIN-STIMULATED ADENYLATE CYCLASE ACTIVITY IN THE RAT HEART

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ABSTRACT

In male rats of three different ages (Group I: 7 weeks, Group II:4 months, Group III: >10 months) basal and forskolin-stimulated adenylate cyclase (AC) activity were investigated in rat heart ventricles of animals sacrificed at 8 time points of within 24 hours of a day. Maxiumum (Emax) and half-maximum (EC_{50}) stimulation by a water-soluble forskolin (FOR; 0.1 - 100 µmol/l) were determined from dose-response curves. In young rats (Group I) significant rhythmicity was found in basal and FOR stimulated AC activity, which was successively reduced or abolished with age by a reduction in amplitude. Mean basal AC activity increased about 2-fold from Group I to Group II and III. Mean maximum in FOR stimulation was about 12-fold in Group I and about 7-fold in Group II and III. EC_{50}-values did neither display a significant rhythmicity in either group nor an age-dependency. It is concluded that stimulatory potency of the catalytic sub-unit of the AC is reduced with age. The mechanisms of the loss in rhythmicity need further elucidation.

KEY WORDS

Adenylate cyclase - rat heart - forskolin - rhythmicity - age-dependency

INTRODUCTION

In earlier studies a daily rhythm was found in the basal formation, content and hydrolysis of cAMP in rat heart (Lemmer et al., 1987). Also in vitro stimulation via the β-receptor by isoprenalin, via the G-protein by Gpp(NH)p and via the catalytic unit of the adenylate cyclase by forskolin displayed significant daily variations (Lemmer and Witte, 1989). It was concluded that the circadian rhythmicity in AC activity was mainly regulated by changes in the properties of the enzyme itself. Recently, diminished NaF-, Gpp(NH)p- and FOR-stimulated AC activities were described in aged rat myocardium, which were attributed to an impaired G-protein and catalytic unit activity in senescence (O'Connor et al.,1983). However, no data are available concerning age-dependent changes in circadian rhythmicity in AC activity. This may be of special interest, because reduction in amplitude and/or loss in rhythmicity can occur in old age (Touitou et al.,1986). Since we recently reported on a pronounced reduction in basal as well as FOR-stimulated AC activity in post-mortem hippocampi of Alzheimer

patients (Ohm et al., 1989) studies in rats of different ages were designed to evaluate whether or not rhythmicity in AC activity is influenced by age.

MATERIAL AND METHODS

Male Wistar rats (Bor: WISW, SPF Cpb) at three different ages (Group I: 7 weeks, about 150 g; Group II: 16 weeks, about 360 g; Group III: > 10 months, about 400 g) were sacrificed at 3-hour intervals within 24 hours of a day during 2-23/24-1989 (I, II) or 3-10/11-1989 (III). They were synchronized to a light-dark schedule of 12:12h [L:7-19h, D:19:7h] with food and water ad lib. Basal and forskolin stimulated adenylate cyclase [AC] activity were determined by measuring formation of cAMP from ATP in the presence of an ATP-regenerating system by radioassay as described (Lemmer and Witte, 1989). Three to five ventricles were studied for each time point. Water-soluble forskolin was used at 0.1, 1, 10, 30 and 100 µM. The dose-response curves were analyzed by a non-linear fitting method (Witte et al., 1989), maximum in stimulation [Vmax] and half-maximum stimulatory concentration [EC_{50}] were calculated. Differences in circadian phase-dependency in basal and FOR-stimulated AC were tested by Cosinor (Witte et al., 1989) and ANOVA.

RESULTS

Fig.1 shows representative dose response curves for the stimulation of the AC by FOR. From these curves the fitted basal AC activity, the maximum in FOR
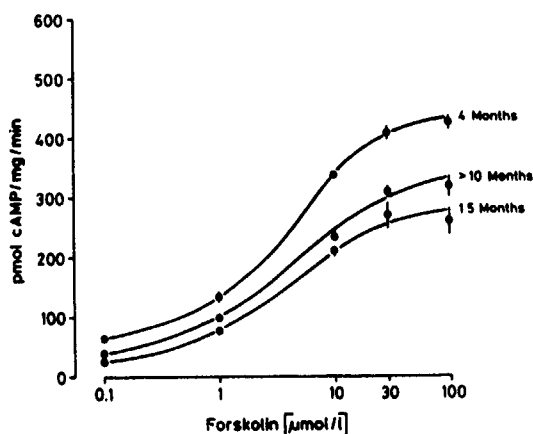
FIG.1. Dose-response curves of the stimulation of the cardiac adenylate cyclase by forskolin in rats of different ages.

stimulation (Vmax) and the half-maximum in FOR stimulation (EC_{50}) were calculated by non-linear fitting (Witte et al., 1989). The values in basal and Vmax of FOR-stimulated AC activity obtained in the 3 groups of rats within 24 hours are shown in Fig. 2 and 3, demonstrating an age-dependency in basal AC activity in rat heart ventricles. Analyses of data on basal and FOR stimulated AC activity are compiled in Table 1. It can be seen that both basal and Vmax in FOR stimulated AC activity displayed a significant rhythmicity (12-hour rhythm) in young rats, but not in 4 months aged animals nor in old rats. Amplitude after FOR stimulation in Group II and III animals was reduced by about 55% when compared to that found in Group I (Table 1). Basal AC activity increased about 2-fold with age in Group II and III. On the other hand maximum stimulation by FOR above basal values was reduced in group II and III rats (about 7-fold)

Fig.2. Basal AC activity in rat
heart ventricles of rats of
different ages (I=7 weeks, II=4
months, III=>10 months), mean
values ± SEM, n=3-5.

Fig.3. Maximum stimulation by
forskolin of the cardiac adenylate
cyclase in three groups of rats
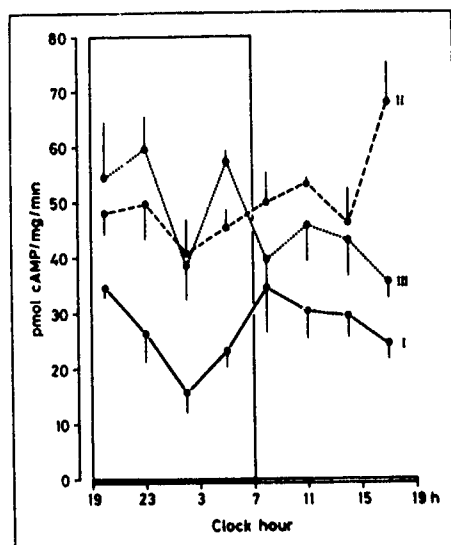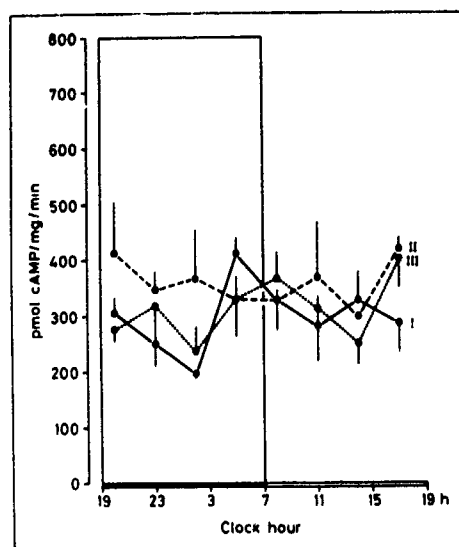(I=7 weeks, II=4 months, III=>10
months), mean values ± SEM,
n=3-5.

in comparison to the young rats of Group I (about 12-fold) (Table 1). EC_{50}-values in FOR stimulation were in the range of 2.0-7.4 µmol/l in the three groups of rats and within 24 hours, in neither group significant daily variations were found. A Hill coefficient of about 1.0 was found in all experiments, indicating that there is a single class of binding sites.

Group	Mesor [pmol/mg/min]	Amplitude	Acrophase [hr]	tau [hr]	Significance p Cosinor	ANOVA
ADENYLATE CYCLASE (mean±SEM)						
Basal Activity.						
I	26 8± 1.5	6 9± 2.2	8.61±0.54 20 51±0.54	12	0 014	<0.05
II	49.7± 2 1	5.8± 2.9	15.61±1.91	24	n s.	<0 05
III	46 3± 2 8	5.1± 3.9	9.42±1.47 21.42±1.47	12	n.s	n.s
Forskolin Stimulation:						
I	303 4±15.5	66.1±21.3	5 96±0.66 17.96±0.66	12	0.015	<0.01
II	359.8±23.2	37.8±32.8	20 28±3.32	24	n.s.	n.s.
III	307.3±20.0	38.9±28.2	7 21±1.39 19.21±1.39	12	n.s	n s

Tab.1. Cosinor analyses of adenylate cyclase activity (basal and maximum stimulation by forskolin) in rat heart ventricles of rats of different ages, I=7 weeks, II=4 months, III=>10 months.

DISCUSSION

The results clearly demonstrate for the first time that the rhythmic variation in basal and FOR stimulated AC activity is age-dependent. Already at the ΄.ge of 4 months rhythmicity is nearly abolished. That qualitative changes in AC occur with age can also be taken from the increase in basal AC activity as well as from the reduction in FOR stimulation of the catalytic unit of the AC. At present it is not known which mechanisms are involved in the rhythmic organisation of the cardiac AC. A reduction in AC stimulation in rat myocardium with age was also observed by O'Conners et al. (1983), who also found an EC_{50} of about 3 µM for FOR (non-water-soluble) and one class of binding sites. Our data moreover indicate that the enzyme itself must be changed qualitatively with age, because receptor or G-protein mediated AC stimulation are not involved in the action of FOR.

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EFFECTS OF CAFFEINE ON CIRCASEPTAN RHYTHMICITY FOLLOWING SURGERY IN LABORATORY RATS

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ABSTRACT

Twenty-four male rats were implanted with radio-transmitters. After recovery, core temperature was monitored with radio-telemetry. Circadian and circaseptan rhythms were analyzed during recovery in rats pretreated with caffeine and in control animals. Data were analyzed using cosinor analysis and comparisons between groups were made with binomial frequency distribution. Fewer rats that received caffeine developed circaseptan rhythms than did control rats suggesting that caffeine reduces circaseptan rhythms following surgery. (Supported by DHHS 1 RO1 NU01098).

KEYWORDS

Caffeine, circaseptan, surgery, chronobiotic cues

INTRODUCTION

Human beings may have adapted their activity schedules to accommodate endogenous circaseptan periodicity (Ahlers, 1988). Levi and Halberg (1982) suggested circaseptan "reactive" periods that underlie the surveillance aspects of the body's defense system and presented evidence of the circaseptan oscillation of the body's defense and neural, hormonal, and cellular repair systems Amplification of circaseptan rhythms due to single stimulus induction has been reported following antigenic or traumatic stimulation (Levi & Halberg, 1982), allograft rejection (Ratte et al., 1973), wound healing following maxilla-facial surgery (Pollmann, 1984), balneotherapy (Hildebrandt & Geyer, 1984), and administration of analgesics for surgically related pain (Pollmann & Hildebrandt, 1987). Halberg et al. (1981) concluded that circaseptan rhythms played an important role in surgical outcomes, and that chronobiology needed to be considered when planning surgical care. Hildebrandt and Geyer (1984) suggested that the development of circaseptan periodicities is an adaptation to stress. Ehret et al. (1975) suggested that caffeine was a chronobiotic. He also recommended its use to reduce jet-lag (Ehret et al., 1981). Caffeine's effects on disruption of rhythms during recovery from surgery has been examined (Farr et al., 1985; 1989). In conjunction with these studies, circaseptan rhythms were compared between animals receiving caffeine before surgery and untreated controls.

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MATERIAL AND METHODS

Twenty-four male Sprague-Dawley rats were implanted with radio-transmitters (Mini-Mitter, Co.). Following one month, recovery was confirmed by comparison of the rats' activity patterns with unoperated animals. Rats were randomly assigned to experimental and control groups and housed individually in a light and temperature controlled room with food and water ad lib. The animals were removed from exposure to female rats at weaning, and no other rats were housed anywhere in the building during the experiment. Core temperature and locomotor activity were measured continuously and recorded every 10 minutes using radio-telemetry (DataQuest III, Mini-Mitter, Co.) under a 12:12 LD cycle (lights off at 2000). Baseline data were recorded for at least 16 days. The next 5 days, 12 rats were fed caffeine in distilled water (7.5 mg/kg body weight), and 12 rats received distilled water alone, orally, each day at 2000 hours. Solutions were given with a stainless steel feeding needle and syringe. On the sixth night, abdominal surgery was performed under general anesthetic (87 mg/kg ketamine hydrochloride & 13 mg/kg xylocaine i.m.) at 2000 hours. The rats were then recorded for 27 days during their recovery. Data were analyzed for circaseptan rhythmicity using cosinor analysis (cosine regression), and comparisons were made between groups with binomial frequency distribution.

RESULTS

Four of 11 control animals had significant circaseptan rhythms ($p < 0.05$) during baseline measurement. Individual acrophases occurred on days 2, 3, 5, and 6. During the first 2 weeks of recovery, all 11 control rats developed significant circaseptan rhythms ($p < 0.05$); circaseptan acrophases were clustered (2 on day 2, 8 on day 3, 1 on day 4). Acrophases were more synchronized than circaseptan acrophases during baseline. The second 2 weeks of recovery, only 2 of the 11 rats continued to be circaseptan. Their acrophases were no longer synchronized. During baseline, 2 of 12 caffeine rats displayed circaseptan rhythms ($p < 0.05$). Their circaseptan acrophases occurred on days 1 and 7. The f rst 2 weeks after surgery, 4 additional rats that received caffeine before surgery developed significant circaseptan rhythms ($p < 0.05$) for a total of 6. One rat's acrophase occurred on day 1, 3 on day 2, 1 on day 3, and 1 on day 5. The synchronization noted in control animals was not observed in those that received caffeine. In the final 2 weeks of recovery, only 1 rat that received caffeine continued to display significant circaseptan rhythms ($p < 0.05$). Significantly fewer animals that received caffeine developed circaseptan rhythms than did control animals (Fig. 1). The number of caffeine rats that displayed circaseptan rhythmicity increased by 33% following surgery as compared with a 64% increase in the number of control rats with circaseptan rhythms ($z - 2.79$, $p > 0.05$). Both experimental and control animals underwent 2-3 days of circadian dysrhythmia followed by 2 weeks of lengthened periods. Circadian acrophases of caffeine rats were not as displaced as those of control animals.
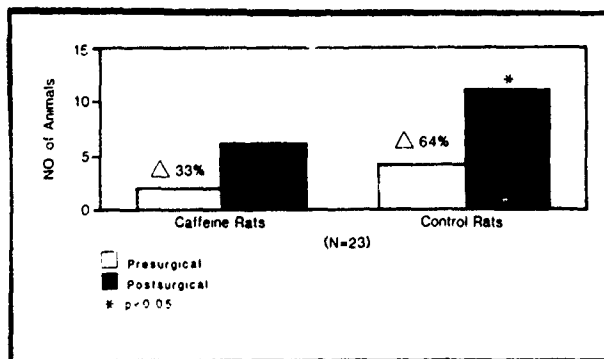

Fig. 1. **Rats with significant circaseptan periods before and after abdominal surgery.**

DISCUSSION

Data from the laboratory rats supports human studies (Hildebrandt & Geyer, 1984; Levi & Halberg, 1982; Pollmann & Hildebrandt, 1987) in which surgery promoted the development of circaseptan rhythms. In addition, untreated animals that developed circaseptan rhythms tended to cycle with synchronized oscillations after surgery. This may be evidence of the single stimulus induction suggested by Levi and Halberg (1982). When circaseptan rhythms were compared with circadian cycles in the same animal's data series, development of circaseptan rhythms did occur at the expense of circadian oscillations.

The data suggested that caffeine ingested early in an animal's active period inhibited the development of circaseptan rhythmicity following surgery. These experiments have since been extended giving caffeine during the animals' middle and late active periods (unpublished data). Caffeine ingested at these times also inhibits the development of circaseptan rhythms, although to a lesser degree.

The question remains whether the development of circaseptan rhythms following surgery or traumatic and stressful events is beneficial. The circaseptan nature of post-surgical deaths (Frey, 1929, reported in Ahlers et al., 1988), as well as the circaseptan cycling of human and murine rejection episodes reported by H _berg et al. (1981) would suggest that an individual recovering from surgery may be at risk during specific phases of the circaseptan cycle. When locomotor activity levels of rats that developed circaseptan rhythms were compared with those that did not, no differences in recovery of activity between the two types was seen. Differences in recovery were observed, however, between those that had received caffeine and those that had not (Farr et al., 1989). Stability of circadian rhythms was increased after surgery when caffeine was given early in the active-phase before surgery (Farr et al., 1985; Farr et al., 1989).

Ingestion of caffeine, a recognized chronobiotic substance, early in the active period appears to influence the development of circaseptan rhythms in rats. This finding underscores the importance of determining whether circaseptan rhythms are adaptive and necessary following surgery. If circaseptan rhythms are detrimental to recovery, then timed ingestion of caffeine could be a useful tool in reducing their influence. On the other hand, if development of circaseptan rhythms is beneficial, depression of these rhythms must be weighed against the effectiveness of caffeine as a promotor of circadian stability following surgery.

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SEX RELATED DIFFERENCES IN ADAPTATION TO CHRONIC CALORIC RESTRICTION IN OLD $B_6C_3F_1$ MICE: IMPLICATIONS FOR CHRONOPHARMACOLOGY AND CHRONOTHERAPY

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ABSTRACT

The circadian rhythms for several physiological and behavioral variables
were measured in male and female $B_6C_3F_1$ mice that were fed ad libitum (AL)
or fed a caloric restricted diet (60% of AL) during the light phase (LF) or
during the dark phase (DF). With a few exceptions, male and female mice
adapted to caloric restriction (CR) in a similar fashion. Compared to LF
feeding, DF feeding was more effective in synchronizing the rhythms between
CR and AL mice and the feeding synchronizer was found to be dominant over
the photoperiod synchronizer for all of the variables measured. The
results of this study suggest that it may be important to synchronize the
delivery of drugs to the meal schedule, especially in subjects that are
calorically restricted.

INTRODUCTION

Chronic CR is associated with increased maximum achievable life span (McCay
et al., 1935) and a decreased or delayed onset of chronic diseases in many
species of mammals (Walford et al., 1974). However, little is known about
the primary mechanisms by which CR interacts with environmental factors to
alter the timing of the biological clock. The specific aims of this study
were (1) to determine if sex-related differences in physiological and
behavioral performance occur in CR and AL mice; (2) to determine the rela-
tive importance of photoperiod and feeding as environmental synchronizers;
and (3) to determine the optimum feeding regimen for CR studies.

MATERIALS AND METHODS

Male and female $B_6C_3F_1$ mice were divided into a control group that was fed
AL and a CR group that received 60% of the AL ration starting at 14 weeks

of age. Ten AL and CR mice (28-29 months of age) were individually placed
in test cages and conditioned to LD 12:12 (lights on from 05:00 and 17:00
hours). Initially, all animals were fed at 10:00 hours daily, 5 hours
after lights on (HALO), during the light phase of the photoperiod cycle.
Physiological performance was continuously measured for a 10-day interval.
Then the AL and CR animals were phase-shifted for 30 days so that they were
fed their respective rations at 17 h·LO during the dark phase of the
photoperiod cycle. CR animals had free access to the feeder at all times
and food was left in the feeder until eaten. Physiological performance was
again measured for another 10 day interval under this regimen. The equip-
ment and testing procedures used in this study were similar to those used
previously (Duffy et al., 1987). The parameters listed in the tables were
continuously and noninvasively measured by an automated data acquisition
and process control system.

RESULTS

Results of multiple T-test analysis comparing old male and female $B_6C_3F_1$
mice are summarized in Table 1. A comparison between sexes in AL mice
revealed that the number of feeding episodes per day and the average body
temperature were higher in males than in females; average food consumption
per episode and RQ range were higher in AL females than in AL males. Male
and female DF and LF restricted mice of both sexes expressed fewer feeding
episodes but consumed more food per episode than their AL counterparts. In
LF caloric restricted mice, daily water consumption per gram lean body
mass, number of feeding episodes per day, and average body temperature per
day were found to be higher in males than in females; average food consump-
tion per episode, and daily body temperature range were higher in females
than in males. Similarly, the number of feeding episodes per day and the
average total motor activity per day were higher in male DF caloric
restricted mice than in female DF mice; average food consumption per
episode and average body temperature range were higher in female DF mice
than in male DF mice. Average body temperature was found to be lower in CR
mice of both sexes than in AL mice, and average activity was found to be
greater in CR mice than in AL mice. Male and female DF and LF restricted
mice expressed fewer feeding episodes but consumed more food per episode
than their AL counterparts.

Chronic CR in the present study is associated with a significant increase
in the amplitude of the circadian rhythms for many of the variables
measured (temperature, RQ, metabolism, activity, etc.) as was reported
previously in rats (Duffy et al., 1989) and in mice (Nelson and Halberg,
1986). Although the circadian rhythms for LF caloric restricted mice were
shifted out of phase with DF caloric restricted and AL mice, in most
instances, the variation in acrophase among various measures within a given
treatment group was small. The results of population mean cosinor analysis
are summarized in Table 2. Significant differences in circadian variables
were found between male and female mice for feeding activity, water
consumption, and food consumption. Acrophase for these variables occurred
earlier in LF male mice than in female LF mice, whereas acrophase for
temperature occurred earlier in female LF mice than in male LF mice. No
significant difference in the timing of acrophase was found between the two
sexes for any of the parameters in AL and DF mice. The amplitude of the
rhythms for feeding activity, water consumption, body temperature, and
oxygen consumption was greater in male AL mice than in female AL mice. In
LF mice, the amplitude of the rhythms for food and water consumption, body
temperature, and oxygen utilization was greater in females than in males.
In DF mice, the amplitude of the rhythms fo drinking activity and food
consumption was greater in males than in females, whereas the amplitude of
the rhythms for water consumption, body temperature, oxygen consumption,
and carbon dioxide production were greater in females than in males.

TABLE 1: B$_6$C$_3$F$_1$ MICE, RESULTS OF T-TEST ANALYSIS

MEASURE (UNIT)	SEX	AL MEAN (SE)		LF MEAN (SE)		DF MEAN (SE)	
Food Consumption (g^{-1} LBM)	M	0.19	(0.00)	0.15	(0.00)	0.13	(0.00)
	F	0.20	(0.00)	0.16	(0.00)	0.15	(0.00)
Water Consumption (g^{-1} LBM)	M	0.13	(0.01)	0.23	(0.05)	0.17	(0.00)
	F	0.15	(0.01)	0.19	(0.01)*	0.18	(0.01)
Avg Food Consumption/Episode (g)	M	0.31	(0.01)	1.20	(0.09)	0.81	(0.02)
	F	0.35	(0.03)*	1.43	(0.07)*	1.12	(0.10)*
Number Feeding Episodes/Day	M	16.51	(0.69)	2.80	(0.23)	4.23	(0.36)
	F	14.12	(1.12)*	2.29	(0.10)*	2.65	(0.18)*
Avg Activity/Day (pulse/hr)	M	10.54	(2.30)	18.26	(1.71)	26.50	(5.57)
	F	9.65	(1.98)	15.06	(2.74)	14.13	(2.28)*
Avg Body Temp/Day (group avg, $^{\circ}$C)	M	36.78	(0.08)	35.54	(0.15)	35.11	(0.18)
	F	36.61	(0.06)*	34.92	(0.34)*	35.14	(0.17)
Avg Body Temp/Hr (24-hr range, $^{\circ}$C)	M	(2.18)		(5.91)		(4.59)	
	F	(1.94)		(10.47)		(6.86)	
Avg O$_2$/Day (g LBM) (ml g^{-1} hr^{-1})	M	3.34	(0.16)	3.44	(0.16)	3.19	(0.06)
	F	3.43	(0.12)			3.29	(0.09)
Avg RQ/Day	M	0.91	(0.01)	0.89	(0.01)	0.89	(0.02)
	F	0.92	(0.01)			0.89	(0.02)
RQ Variation/Day (24-hr range)	M	(0.10)		(0.20)		(0.24)	
	F	(0.12)				(0.25)	

LBM = Lean body mass; AL= Ad libitum; LF = Caloric restricted mice fed during the light period; DF = Caloric restricted mice fed during the dark period; M = Male mice; F = Female mice; * = Significant difference between males and females (P < .05)

TABLE 2: B$_6$C$_3$F$_1$ MICE, RESULTS OF POPULATION MEAN COSINOR ANALYSIS

MEASUREMENT (UNIT)	SEX	ACROPHASE IN HALO			AMPLITUDE		
		AL	LF	DF	AL	LF	DF
Drinking Activity (pulse s^{-1})	M	15.47	7.00	19.66	0.44	0.50	0.71
	F	15.60	7.60	19.10	0.38	0.48	0.50
Feeding Activity (mv s^{-1})	M	15.73	6.93	19.47	0.01	0.01	0.01
	F	15.47	7.60	19.06	0.01	0.01	0.01
Water Consumption (mg s^{-1})	M	15.53	6.60	18.60	1.02	1.59	2.24
	F	16.00	7.50	18.40	0.68	2.32	5.94
Food Consumption (mg s^{-1})	M	15.80	6.73	19.20	0.93	1.17	2.00
	F	15.27	7.60	18.87	0.85	1.88	1.23
Temperature ($^{\circ}$C)	M	16.67	9.66	19.27	1.13	2.40	2.63
	F	16.00	8.60	19.73	0.87	5.21	3.30
Total Activity (pulse s^{-1})	M	17.07	6.33	17.27	9.17	11.93	36.11
	F	17.27	5.33	6.93	8.49	23.78	24.74
Oxygen (ml g^{-1} hr^{-1})	M	17.27	9.47	19.80	0.09	0.15	0.13
	F	17.00		19.93	0.07		0.17
Carbon Dioxide (ml g hr^{-1})	M	17.66	9.93	20.73	0.12	0.16	0.16
	F	17.20		20.67	0.10		0.19
Respiratory Quotient	M	18.60	11.93	23.87	0.06	0.08	0.12
	F	17.60		24.06	0.05	0.13	
Group Mean (S.E.)	M	16.644	(.365)	8.287	(.665)	19.763	(.601)
Group Mean (S.E.)	F	16.370	(0.290)	7.37	(0.440)	19.64	(0.650)

AL = Ad libitum; LF = Caloric restricted mice fed during the light period; DF = Caloric restricted mice fed during the dark period; CI = 95% Confidence interval; M = Male mice; F = Female mice; * = Significant difference between males and females (P < .05)

DISCUSSION

The results of this experiment largely support those found in a previous study in rats (Duffy et al., 1989) which reported that, with a few exceptions, old male and female rodents adapt to LF and DF caloric restriction in a similar fashion. The fact that sex differences in the timing of acrophase were found for several measures in LF mice but not in DF mice, and that AL animals were highly synchronized to DF mice but were out of phase with LF mice, may suggest that feeding during the dark phase rather than during the light phase is the more normal feeding regimen and is therefore the optimum caloric restriction regimen. When feeding was out of phase with the photoperiod rhythm, as was seen in the LF regimen, acrophase for all of the measures shifted to occur at or around the feeding time, which may indicate that in caloric restricted mice the feeding synchronizer is dominant over the photoperiod synchronizer.

The reason why daily mean values and certain acrophase and amplitude values for several of the variables are different between male and female mice is unknown. A possible explanation is that because of their smaller body size and surface area, female mice may have a higher rate of thermoconductivity resulting in increased heat loss and greater metabolic and thermoregulatory requirements. However, considered in their entirety, these sex-related changes were significant but may not be of any great importance.

The results of this study have several important implications relating to chronopharmacology and chronotherapeutics. It is important to synchronize the delivery of drugs to the meal schedule, especially in subjects that are caloric restricted. Even when feeding is not synchronized between treatment groups in CR studies, in some instances the results of an experiment can be normalized relative to the dominant environmental factor (presentation of food) by expressing data in hours after feeding acrophase rather than in clock time. Additional studies are needed to investigate the interaction between drug intervention and nutritional parameters.

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INFRADIAN AND CIRCADIAN VARIATION IN URINE VOLUME AND ANALYTES OF A COMATOSE POST CVA WOMAN RECEIVING CONTINUOUS NOURISHMENT OVER 11 MONTHS

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INTRODUCTION

It has been documented that aging is accompanied by changes in circadian rhythm characteristics (Miles, 1980; Minors, 1981; Casale, 1984; Swoyer, 1989). The most notable changes occur in the shift of acrophase and in the decline in amplitude. A recent study on variation in meals and sleep patterns in subjects 50-92 years of age revealed that increasing age was correlated with decreased variability in retiring, rising and eating meals, earlier sleep times, increased frequency of daytime naps and nocturnal awakening and decreased physical activity (Minors, 1989). These external events all influence observed circadian variations.

The data presented in this report are of a 90-year-old woman, completely incapacitated by CVA who remained comatose and periodically semi-conscious. She was nourished continuously at a constant rate through a G-tube from August 14, 1988 until her death on October 2, 1989. Twenty-four hour urine outputs were measured for 336 consecutive days between the months of September 14, 1988 and August 15, 1989. This data series provided a unique opportunity to statistically analyze the daily rates for any underlying infradian periodicities which were present in the absence of most, if not all, masking effects of the environment (climatologic, dietary, social and/or sleep-wake related)(Minors, 1989). In order to characterize the circadian variation in urinary analytes, fractionated 3-hr urines were also collected on Sept 14 and Oct 14, 1988. The 8 samples from each circadian collection were analyzed for Na, K, Ca, Mg, P, Zn, Si, Al, B, urea nitrogen, creatinine, uric acid, pH, total solids, total proteins, epinephrine, norepinephrine and dopamine.

SUBJECT, MATERIALS and METHODS

Subject Prior to May 9, 1988, the day of cerebrovascular accident (CVA) with left-sided paresis and hospitalization, this 90-year old woman (M.T.) was healthy, alert, with a warm, friendly disposition. She was well nourished, well developed, 5ft 4in (163 cm) tall and weighed 154lbs (70 kg). Her history was significant for coronary artery disease and hypertension and was marked with rigorous non-compliance to medications. She ate very little red meat and especially liked complex carbohydrates (pasta) and fruits (grapes, pears, figs, apples, grapefruit, etc). She ate very little and was "picking" frequently throughout the day. She was also "cat-napping" frequently throughout the daytime hours and her sleeping periods extended into late morning hours. She was a habitual black coffee drinker, a habit which was increasing with intensity and age until her CVA on May 9. On hospital admission her blood pressure was 150/80 mmHg, heart rate 80 bts/mn, and temperature 100.8°F (38.2°C). On May 19th urine analysis was within the normal range. Hematology results were also within normal ranges as follows: RBC: 4,370,000; Hgb: 11.6gm; WBC: 9,800 with a normal differential and platelets: 419,000. Serum chemistries examined by SMAC-24 for electrolytes and liver enzymes (CPK assays were not done), were within normal ranges. Other values (mg/dl) were: cholesterol: 117; triglycerides: 163; P: 4.2; glucose: 113.

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Upon hospitalization she required a nasal gastric tube because she refused all oral feedings. On July 12, a #12 French feeding tube was inserted into the proximal small intestine. She was transferred home on August 5, where she was cared for by her two daughters over the ensuing 14 months as she remained bed-ridden and incontinent. Urine was excreted through the Foley tube and fecal excreta collected in a diaper. She became less and less responsive to the stimuli of voice and touch, remaining comatose and only occasionally semi-conscious until her death on October 2, 1989. According to her attending physician she may have had a number of small additional CVAs. During this entire period of time she did not appear to have lost any weight. She did not have a single bed sore. Her skin remained warm and dry and had good turgor - a tribute to the love and care provided by her two daughters.

Material The administered food consisted of an aqueous preparation made up of PROPORTION (Modular Protein Supplement) and TOLERAX (Nutritionally complete elemental diet). This mixture provided a nutritionally complete diet formulated for the management of patients with impaired digestion. The daily feeding consisted of 1267 calories administered via electrical pump into the small intestine at the rate of 70 ml/hr (1680ml/24hr). The daily dose contained the following: protein: 25gm (USDA= 45gm/dy); amino acids nitrogen: 3.7gm; fat: 4.32gm; carbohydrates: 259gm; Na: 786mg; K: 2137mg; Ca: 1682mg; Mg: 642mg; P: 630mg; with essential vitamins and trace elements.

Methods Twenty-four urines were collected from 1600 on one day to 1600 on the next through a Foley tube into a plastic container. The collection was transferred into a 1 liter graduated cylinder and the volume recorded to the nearest 5 ml. These collections were made across 336 consecutive days beginning Sept 14, 1988 and ending Aug 16, 1989. Because the fluid intake (1680 ml/24hr) was continuous and constant, the daily urine excretion could be expressed as % of daily input. The data were edited prior to analysis. On 4 occasions, 24h output was <10% of input in the presence of a wet diaper due to tube leakage (on Nov 29, Dec 21, Dec 28 and Jan 5) and these values were excluded. On 2 other occasions (Nov 15 & 18) output was <7% of input with tube blockage listed as a probable cause. All data were subsequently filtered by computing 2-day moving means, resulting in 335 values for analysis. The daily urine volumes were analyzed for infradian periodicities by the linear least squares (LLS) fitting of cosines with periods ranging between 3 and 365.25 days with 1-d increments between trial periods (Nelson, 1979). Periods significant at p≤0.05 by LLS were subjected to nonlinear LLS (NLLS) analysis to derive 95% limits of the period (DePrins, 1986).

In order to describe the 24h time structure in several urinary analytes, fractionated 3-h urines were collected around-the-clock on 2 occasions (Sept 14-15 and Oct 14-15, 1988). The 8 samples from each circadian collection were analyzed by conventional methods for: pH, Na, K, Ca, Mg, Zn, Si, B, urea nitrogen, creatinine, uric acid, P, total solids, epinephrine, norepinephrine and dopamine. Total proteins were determined using the Coomassie Brilliant Blue G-250 stain (using reagents purchased from Bio-RAD, Chemical Division, Richmond, CA 94804). Circadian variation of each 24h profile was assessed by the range of change (ROC = difference from lowest to highest value) and by the LLS fit of cosines ranging from 20 to 28 h with 0.1h between trial periods. Resulting parameters were compared (Bingham, 1982). No data were excluded or filtered in these analyses.

RESULTS and DISCUSSION

Results The original values for 24h urinary output are presented in Fig 1. LLS analysis of 2-day moving means resulted in detection of 6 trial periods at p≤0.05, as shown in Table 1-left. The composite waveform from these 6 components can be seen in Fig 2. A linear trend was not present (p=0.148), thereby suggesting that the observed circannual variation is an endogenous component with a peak in the Spring. The six candidate infradian periods detected in 24h urine output (365,

Table 1: Linear-nonlinear Least Squares Analysis of Daily Urine Output by a Comatose CVA Woman (MT, 90-91y) Nourished Continuously at a Constant Rate (1680ml/24 hours)*

Results: *LINEAR LEAST SQUARES Analysis*								*NON-LINEAR LEAST SQUARES Analysis*				
Trial Period								Period				
(hrs)	(days)	%R	p value	Amp	se	Ø	(95% limits)	(days)	(95% limits)	Amp	(95% limits)	Ø (95% limits)
8766	365.25	18.6	<0.001	6.9	0.8	-243°	(-229,-257)	423.6	(358.8, 576.9	7.0	(3.1,10.8)	-214° (-178,-252)
2784	116	12.8	<0.001	6.1	0.9	-323°	(-307,-339)	·	·	·	·	·
1488	62	7.7	<0.001	4.6	0.9	-068°	(-47,-90)	62.7	(59.0,68.0)	4.9	(0.8,9 0)	-064° (-12,-114)
528	22	3.6	0.002	3.2	0.9	-016°	(-344,-48)	·	·	·	·	·
1008	42	2.7	0.011	2.7	0.9	-336°	(-299,-13)	·	·	·	·	·
197	8.2	2.2	0.026	2.5	0.9	-027°	(-346,-69)	·	·	·	·	·

*Using 2d moving means across 336d (Sept 14, 1988 - Aug 16, 1989). Daily output expressed as % of 24h input.
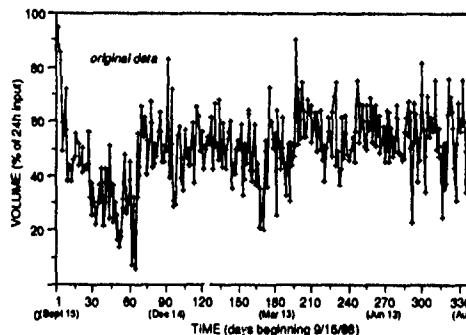KEY: %R=percent rhythm, Amp=amplitude; Ø=acrophase (ref: Sept 15, 1988, 360°=trial period); MESOR=49.7%

Fig 1. Daily Urine Output in Comatose CVA Woman (M.T., age 90-91yrs) Nourished Continuously at a Constant Rate (1680 ml/24h) Over 336 Days
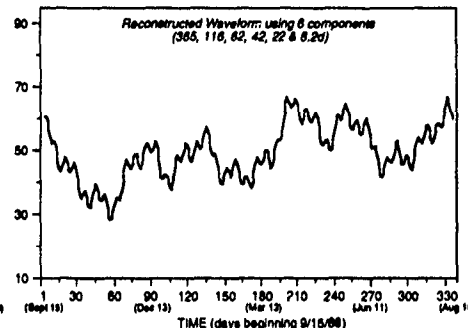
Fig 2. Reconstructed Waveform of Daily Urine Output in Comatose CVA Woman

116, 62, 42, 22 and 8.2 days) were further subjected to NLLS analysis. Two components survived NLLS analysis: 424d (95% limits = 359, 577d) and 63d (59, 68d) (see Table 1-right). Thus, a circannual component with confidence interval overlapping precisely one year (8766h) and a bi-monthly component could be confirmed in this time series. The reconstructed waveform using 365.25 & 62 days can be seen superimposed over the 2d moving means in Fig 3.
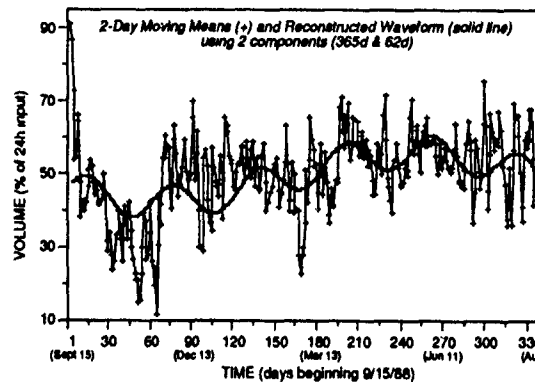

Fig 3: Daily Urine Output in Comatose CVA Woman and Waveform reconstructed from 2 components verified by NLLS

Circadian chronograms revealed considerable variation (generally >50% and as much as several hundred percent) for each analyte in each of the two 24h collection spans. LLS analysis described significant (p≤0.05) circadian rhythms in the Sept profile for Na, K, Ca, Mg, uric acid, Al and pH, but for only epinephrine and pH in the Oct profile. Data characteristics and 24h cosine characteristics in each span are presented in Table 2. No ultradian components, using trial periods of 12 and 8 hours, were detected. Some analytes of interest are presented in Fig 4.

Table 2: Circadian Variation in 3-h urinary analytes (n=8) observed on 2 occasions

Analyte	units	Span1: Sept 14-15, 1988						Span2: Oct 14-15, 1988					
		Low	High	ROC	(%)	Mesor	Amp	Ø	Low	High	ROC	(%)	Mesor	Amp	Ø
Norepinephrine	ug	1.54	3.86	2.32	(151)	2.72	0.26	1928	1.82	4.84	3.02	(166)	3.55	1.00	0448
Epinephrine	ug	0.14	0.73	0.59	(421)	0.36	0.15	1824	0.10	1.03	0.93	(930)	0.56	0.47	0916
Dopamine	ug	3.98	22.60	18.6	(468)	10.48	3.61	1724	4.84	12.68	7.84	(162)	8.86	1.14	0948
Volume	ml	160	390	230	(144)	229	32	2016	55	93	38	(69)	74	7.4	0656
pH		6.64	7.35	0.71	(11)	6.91	0.34	1640	4.96	5.28	0.32	(6)	5.10	0.11	0908
Total Solids	gm	2.79	4.73	1.94	(70)	3.78	0.46	1324	2.50	5.78	3.28	(131)	4.08	0.97	0944
Urea Nitrogen	gm	0.88	1.29	0.41	(47)	1.10	0.42	1208	0.75	1.30	0.56	(75)	1.07	0.16	0328
Creatinine	mg	54	78	24	(44)	68.9	4.1	1016	49	81	32	(65)	66.6	8.9	0616
Uric Acid	mg	22	37	15	(68)	31.4	5.5	1332	23	73	50	(217)	46.3	15.4	0832
Total Protein	mg	16	28	12	(75)	22.4	2.4	1652	25	42	17	(68)	32.5	0.6	0856
Osmolality	mOs	45	81	36	(80)	65.8	12.1	1440	33	65	32	(97)	20.1	8.5	0744
Na	mg	216	521	305	(141)	358	130	1444	20	65	45	(225)	36	17	0932
K	mg	58	223	165	(284)	123	68	1316	49	92	43	(88)	69	13	0816
Ca	mg	16.8	25.8	9.0	(54)	20.5	2.9	0848	14.0	27.3	13.3	(95)	2.5	4.4	0620
Mg	m	4.8	8.3	3.5	(73)	6.4	1.2	0752	3.5	6.2	2.7	(77)	4.9	0.8	0620
Phosphorus	mg	16	28	12	(75)	22.4	2.4	1652	25	42	17	(68)	32.5	0.6	0856
Zinc	ug	12	95	83	(692)	31.4	21.7	1252	64	115	51	(80)	95.9	12.6	0504
Silicon	mg	0.56	1.03	0.47	(84)	0.85	0.19	0916	0.78	1.30	0.52	(67)	1.05	0.15	0552
Aluminum	ug	1	104	103	(>999)	55.0	40.3	1200	46	71	25	(54)	58.0	5.6	1048
Boron	ug	27	100	73	(270)	46.0	19.6	1628	14	24	10	(71)	19.0	1.5	0744

KEY: ROC=range of change from lowest to highest value; Amp=amplitude; Ø=acrophase (clock Hr & Mn), ref=0000. Mesors or amplitudes in bold significantly different at p≤0.05 by parameter test.

Discussion Under constant input, a circannual, as well as a 63-day component was found in 24h urinary output. While the circannual component was anticipated, the 63-d one was not. While trying to steer clear of a parallactic view (Enright, 1989), we only report the 63-d period here as a significant component which survived LLS-NLLS analysis and thus is used in describing the waveform of urine output in a comatose victim. Indeed, the 6 components found by LLS contribute to a waveform which seems to the eye to better approximate the original data. While the 8.2-day component is suggestive of a free-running circaseptan periodicity, failure of the NLLS analysis to compute 95% confidence limits of the period does not allow us to accept 8.2-d with true confidence. This period might

Fig 4: Circadian Variation in Urinary Excretions in Comatose CVA Woman Nourished Continuously at Constant Rate (1680 ml/24h) (3-hourly sampling Sept 14-15 and Oct 14-15, 1988)*

have occurred by chance, might be of a very low amplitude or is a resonating frequency of the longer infradian components accepted by NLLS analysis.

Changes in the amplitude of circadian rhythms in aged subjects can arise from several causes, such as a decrease in the peak rate of secretion (for several hormones) or as the result of a raised minimum value of the rhythm such as deep body temperature and plasma norepinephrine, or from a combination of these two factors with sleepiness and/or urinary excretion (Minors, 1989). In the presence of coma, constant diet, inactivity, supine position and advanced age, large changes in 3-hourly analyte excretion rates in two 24h profiles were revealed macroscopically in chronograms with ROCs from 44% to >400% suggesting the persistence of a circadian component in a comatose patient. However, the circadian time series are too short to make conclusions as to any free-running periods and timing of the circadian rhythms. While the acrophases are more scattered and generally in the afternoon in Sept, they have all moved to the morning hours and are more clustered by October. Whether the free-running periods are longer or shorter than 24 hours cannot be ascertained with the limited data at hand. Three hour urines collected over several days would be needed to accurately assess the circadian system in comatose patients, both to describe the true period (if it is indeed free-running and different from 24.0 hours) and to diagnose the synchrony or dyschrony between different analytes.

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SECTION 5

IMMUNOLOGY AND CANCER

Chairpersons: I. E. Ashkenazı and F. Lévi

CIRCADIAN RHYTHM OF INTERLEUKIN I (IL-1) IN SERUM OF HEALTHY MEN

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ABSTRACT

IL-1α and IL-1β serum concentration was measured every 4 hrs for
24 hrs in 6 healthy men using a sandwich ELISA method. IL-1 serum
concentration was under minimal detection level of the test in all
subjects for IL-1β and in 2 subjects for IL-1α. The other 4 had a
peak between 12.30 hr and midnight and a through in the morning. A
2-way analysis of variance validate the differences related to
sampling time ($p < 0.04$) and to subjects ($p < 0.005$), as well as a
sampling time-subject interaction ($p < 0.05$). This indicate
intersubject differences in both 24 hrs-mean and possibly in
waveform. Our results suggest a differential role for IL1-α and
IL-1β in the physiology of the immune system.

KEYWORDS

Immunology; Interleukin I; Circadian variation

INTRODUCTION

Stimulated macrophages secrete 2 peptidic mediators IL-1α and
IL-1β among other molecules (DINARELLO, 1989). Although these 2
molecules show only 25% sequence homology (GUBLER et al., 1986),
they share the same activity through the same receptor (BAGBY
1989). They induce fever, secretion of growth factor by other
cells (interleukin 6, granulocyte/monocyte-colony stimulating
factor etc.), expression of adhesion molecules (ELAM-1, ICAM-1) on
leukocytes (DINARELLO, 1987). They participate to neuro-immune
relations, for exemple they induce anorexia, somnolence and
decreased perception of pain (NETA and OPPENHEIM, 1988). Because
of this and of the prominent circadian organization of the immune
system, we measured the serum concentration of IL-1α and IL-1β.

MATERIALS AND METHODS

6 healthy young men with a diurnal activity from 8.00 hr to midnight and a nocturnal rest participated to this study. Subjects were studied in January (1), in April (3), and in November (2). Blood samples were taken every 4 hours for 24 hours (from 8.30 hr to 8.30 hr the next day). After complete clotting of blood (10 ml), serum was obtained by centrifugation, aliquoted, immediately frozen and strored at -30°c until used. IL-1α serum concentration was dosed using a sandwich ELISA method (IL-1α ELISA, ENDOGEN Inc. BOSTON USA). Intra-assay coefficient of variation was 5%, the sensitivity was 50 pg/ml. IL-1β serum concentration was dosed using a sandwich ELISA method (Interleukin-1β ELISA kit, CISTRON Biotechnology Inc. PINE BROOK USA). Intra-assay variability was 5.3% and inter-assay variability was 9.4%, the sensitivity was 20 pg/ml. Each time series was dosed in the same assay. For each subject, data were expressed as pg/ml and as percentage of the 24 hrs mean, and analysed using cosinor and 2-way analysis of variance (ANOVA).

RESULTS

IL-1β serum concentration was under minimal detection level of the test at each time point in all subjects. IL-1α serum concentration was less than 50 pg/ml at each time point in 2 subjects (1 in April, 1 in November). Although low, the other 4 had detectable IL-1α serum concentration for at least 4 time points. Therefore statistical analysis were performed on these 4 men. There was a great disparity in the IL-1α level between subjects as shown in figure 1.
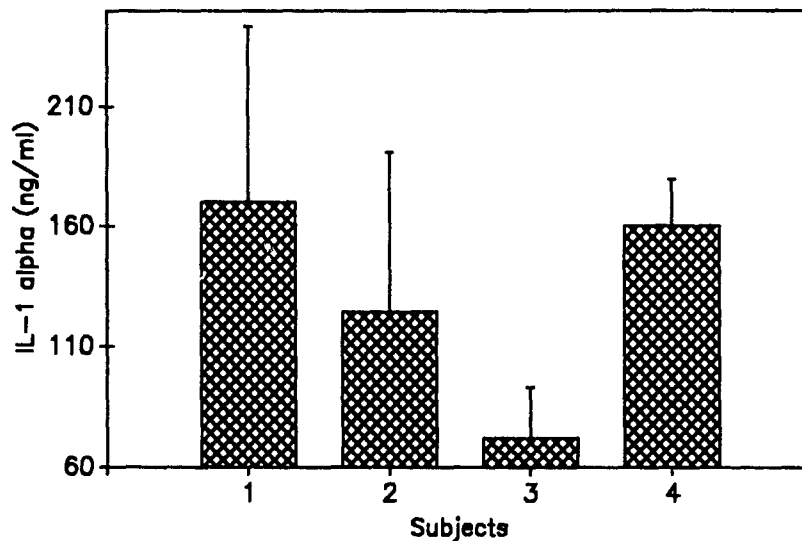

Fig. 1. 24 hr mean serum concentration of IL1—α for each men

All men had a peak between 12.30 hr and midnight and a trough in the morning. When taken together, IL-1α serum concentration of the 4 men showed an evening peak at 20.30 hr (mean±SEM: 173±80 pg/ml) and a through in the morning (mean±SEM at 12.30 hr: 86±51 pg/ml) as shown in figure 2; however cosinor did not detect any rhythm either with a 24 hr or a 12 hr period. Anov. validated differences related to inter-subject variations (p<0.005) and to sampling time (p<0.04), as well as sampling time-subject interaction (p<0.05).
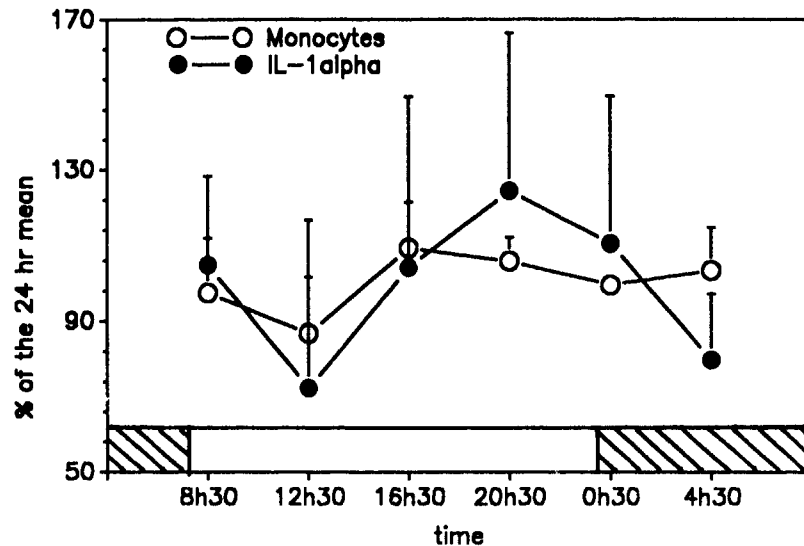
Fig. 2. Chronogram of the % of the 24 hr mean of IL—1α and monocytes

In addition we have determined, for the same 4 men, the counts of circulating monocytes by flow cytometry. It varied from 492 ± 192 cells/mm^3 at 12.30 hr to 604 ± 163 cells/mm^3 at 16.30 hr (see figure 2). Anova validated a sampling time differences but no inter-subject variation.

DISCUSSION

Apart their effects in inflammation and T lymphocytes activation during the immune response to antigens, IL-1α and IL-1β act on hematopoiesis and regulate the adhesion molecules ELAM-1 and ICAM-1 on leukocyte membrane (Bagby, 1989). This may suggest that IL-1 could play a role in the regulation of the immune system at a physiologic level. Thus, we studied IL-1α and IL-1β serum concentration in 6 healthy men.

We found that IL-1β concentration never reach 20 pg/ml even though that of IL-1α could be detected in 4 of 6 men. This is quite surprising since IL-1β concentration is largely higher than IL-1α in supernatant of activated macrophages or in body fluid of

patient during an inflammatory process (Dinarello, 1989). Start
IL-1α serum concentration was low and thus in the less sensitive
part of the titration curve, nonetheless these preliminary results
indicate that IL-1α serum concentration seem to vary in a
circadian manner; and perhaps, like for eosinophils (Whitcomb et
al., 1989), both kind of IL-1 may play different role: IL-1β might
act during pathological process and IL-1α might be involved in the
physiologic regulation of the immune system.

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CIRCADIAN VARIATIONS OF HEMOREGULATORY PEPTIDE EFFECT ON PMN MIGRATION IN MICE

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ABSTRACT

A hemoregulatory pentapeptide (HP) extracted from granulocyte
and described by Paukovits and Laerum is known to have specific
action in the hemopoietic system with a high selectivity for
myelopoietic cell. The present study shows an inhibitory effect
of the peptide on granulocyte migration during inflammatory
process. A circadian rhythm was observed in control and traited
animals. The acrophases were present during the first half of
light span.

KEY WORDS

Hemoregulatory peptide ; granulocyte ; inflammation ; circadian
rhythm ; mice.

INTRODUCTION

The hemoregulatory peptide (HP) originally extracted from
mature human granulocyte, was described in 1982 by Paukovits
and Laerum (11). The peptide inhibits myelopoietic cell
proliferation in bone marrow and reduce the number of
granulocyte in peripheral blood (6,7). The peptide seems to
abolish the circadian rhythmicity both for cell proliferation
and for the recognizable myelopoietic cells (8,10). In
connective tissue when inflammatory process occurs there are
circadian and circannual variations of granulocytes migration
(2,3). The current study was designed to determine whether the
dimer form of the HP was effective on the circadian granulocyte
migration induced by BCG in swiss mice.

MATERIALS AND METHODS

Animals :

179 swiss male mice, 8 to 12 weeks of age (30g) were used
throughout all the experiments. The animals were purchased from
IFFA CREDO, Les Oncins, France. They were housed in an air-
conditioned room (21 ± 1°C) on a L : D 12 : 12 (light on : 0700
- 1900) for at least ten days before experiments. Food and
water were available ad libitum. 9 to 10 animals were divided
into 18 batches.,

Experimental design :

The PMN migration was studied according to the method already
described (1). Briefly, a rayone-made disk (from Ethnor-France)
impregnated with 10 µl of 4 - 6 x 10^6 live BCG suspension / ml
was implanted beneath the skin at 6 different time of the day
(0100, 0500, 0900, 1300, 1700 and 2100). The implant was
removed 480 mn later, fixed in Bouin-Holland's solution,
processed for 5 µm paraffin sections and stained with Groat's
hematoxylin. Five sections of each disk were counted with an
Artec cell counter. The results were presented as a mean number
of PMN ± S.E. / 10000 µm² of the original section.
Thirty minutes before cell trap implantation, each animal
received I.P. either 1,22 µg or 122 ng of HP in 0,1 ml saline
solution. Equal amount of saline was injected in control
animals.
The experiment was performed in April.
Statistical analysis were realized with the Student-t-test and
cosinor analysis.

RESULTS

The peptide inhibits cell migration (mean value = 20,6 cells /
10000 µm², compared to the control group (27 cells / 10000
µm²). High doses of dimer (1,22 µg) significantly reduced the
number of granulocyte (Table 1) from the control value of 54.35
± 8.07 to 36.92 ± 12.97 at 0900 (p<0,01) and also at 1700 from
30.54 ± 11.53 to 15.35 ± 8.98 (p<0,01). With low doses (122 ng)
the peptide is more effective on the number of PMN at 2100,
0100 and 1300 compare to the control (table 1)

Time	Control	HP 122 ng	HP 1,2 µg
0100	7.8 ± 3.8	3.7 ± 2.6**	6.5 ± 3.2
0800	22.8 ± 7.6	17.1 ± 8.1	30.6 ± 10.7
0900	54.4 ± 8.0	47.1 ± 12.7	36.9 ± 12.9*
1300	27.3 ± 7.0	14.6 ± 4.0*	20.6 ± 9.3
1700	30.8 ± 11.5	32.8 ± 15.7	15.3 ± 8 9*
2100	20.2 ± 10.0	4.8 ± 2.2*	13.8 ± 6.1

Table 1 : Number of PMN / 10000 µm² in mice treated with either saline or 122 ng, or 1,22 µg of HP. Significant differences are observed between HF treated and non HP treated animals.
 * p < 0,001
 ** p < 0,02

	Control	Hemoregulatory peptide dimere	
		1.22 µg	122 ng
mice nb	59	60	60
mean level	27.6	20.6	20.0
amplitude	16.1 ± 5.9	12.6 ± 5.0	15.4 ± 6.7
acrophase	11.2 ± 1.4	8.9 ± 1.6	11.0 ± 1.7

Table 2 : Cosinor analysis of PMN migration in control and HP treated mice. (number of cells / 10000 µm² . p < 0,005)

DISCUSSION

The findings demonstrate that PMN migration is inhibited with high and low doses of HP at different time of the day. In previous studies it has been demonstrated that HP inhibits the proliferation of bone marrow stem cells (8). The granulocyte numbers in peripheral blood were also reduced (7).
The HP is also able to inhibit circadian rhythm of cell proliferation (8,10), more over it slows down the whole cell cycle by reducing the entrance into the cell cycle, i.e. the S-phase. During the inflammatory response the circadian rhythm of granulocyte migration is maintained despite the inhibitory effect of the peptide.
Some granulocyte functions had been studied and no effect was seen neither on the kinetics of internalization of Zymosan particles, nor on the saturation density of phagocytosed material (9). In this experiment, granulocytes migration in vivo is significantly dose and time dependant. The strongest

inhibitory effects are observed with low doses at 0900 and 1700
when at 0500 no inhibition was found. With high doses on the
contrary no effect was found at 0500, 0900 and 1700. Same
variations of dose effect are encountered with HP on erythroid
and myelopoietic colony formation in vitro (5) and with
corticosterone on BCG-induced granulocyte migration in normal
and adrenalectomized mice (4).

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CIRCADIAN RHYTHMS OF SURFACE EPITOPE NUMBERS (CD3 AND CD4) FROM CIRCULATING LYMPHOCYTES OF HEALTHY MALE HUMAN SUBJECTS

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ABSTRACT

Circulating lymphocytes and their T subsets including pan T(CD3) and helper T(CD4) lymphocytes exhibit a circadian rhythm (T ≡24 hr) with a peak in the middle of the night. Such circadian organization is seasonally-modulated. The circadian peak-trough difference in total, T(CD3) and T(CD4) lymphocyte count vary from 0 in March (no rhythm) to 46-68% of the 24-hr mean in November (J. Clin. Invest. 1988, 81, 407-413). In a subsequent study, 5 healthy male subjects (26-33 y.o.) had blood drawn every 4 hours for 36 hours in March 1987. Lymphocyte subsets and epitope density were determined by flow cytometry (Epics C, Coultronics). The lack of any group-synchronized circadian rhythm was confirmed in this season for the counts of total, T(CD3) and T(CD4 lymphocytes. Nonetheless, the numbers of CD3 and CD4 molecules at the surface of T(CD3) and T(CD4) cells exhibited a circadian rhythm with a peak at 08.00 hr. The number of CD3 molecules doubled between 20.30 hr (mean= 10,200±620 per cell) and 08.30 hr (mean= 26,000±890 per cell) on the T(CD3) lymphocyte membranes. At night, mean CD4 molecule number was at the limit of detection and reached 10,200±600 at 08.30 hr. At least two different systems of rhythmic regulations appear to characterize immune defences. One is involved into the regulation of the circulation of T(CD3) and T(CD4) lymphocytes and another one modulates the density and/or expression of CD3 and CD4 molecules. An endogenous temporal organization of these two systems could reflect adaptation of living beings to environmental cycles: antigen recognition may be more efficient during the activity phase through an increased number of CD3 and CD4 molecules, whereas proliferation of immune cells and/or their liberation from peripheral organs would occur at night. Circadian rhythms of epitope numbers

(CD3 and CD4) on lymphocyte surface may constitute a
guide for optimizing treatments with biological response
modifiers.

CIRCADIAN STAGE DEPENDENT RESPONSE TO INTERLEUKIN-2 IN MOUSE SPLEEN AND BONE MARROW

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ABSTRACT

The circadian stage dependency of the immune response to interleukin-2 (IL-2) was determined in mice. Significant endogenous circadian rhythmicity (>50% of daily mean) was detected in spleen weight, proportion of T-suppressor cells in spleen and bone marrow, and Lyt-5.2+ (natural killer) cells in bone marrow. IL-2 (25,000 Units/day i.p. x 3 days) increased the number of spleen cells by 16% overall, but up to 3-fold more when it was given during the activity span of the animals. The capacity of IL-2 to modify the proportion of lymphocyte subsets depended upon the circadian stage of administration (e.g., a significant increase of Lyt-5.2+ cells occurred only after treatment during the animal's activity phase).

KEY WORDS

Circadian rhythmicity; interleukin-2; immune-response; natural killer cells.

INTRODUCTION

High amplitude circadian rhythmicity has been found on a cellular and subcellular level, which can affect biochemical reactions as well as regulatory mechanisms (e.g., hormones) in the intact organism (Haus et al., 1988). The circadian integration of the immune system has been described (Levi et al., 1988) and circadian rhythms are therefore anticipated in the immune response to IL-2 as well (Levi et al., 1990). IL-2, previously called T-cell growth factor, is a glycosylated polypeptide produced by T-lymphocytes during an immune response. It initiates the proliferation of activated T-cells and induces or increases the activity of natural killer (NK) cells (Rosenberg et al., 1984). Recombinant IL-2 has been used therapeutically to induce cytotoxic activity which may cause regression of various malignancies (Rosenberg et al., 1987). Our objective was to investigate the circadian pattern of the immune response to IL-2 by first determining the optimal non-toxic dose and treatment schedule of IL-2 and the time interval to maximal response. The distribution of lymphocyte subsets (NK, T-helper or T-suppressor lymphocytes or B-cell phenotypes) was then measured in response to IL-2 administered at one of six different circadian stages and compared to controls who received the drug vehicle only.

MATERIALS AND METHODS

Animals: Female 8-12 week old C_3H mice were adapted to a 12:12 hour lights on:off schedule in an air-conditioned room. Standard laboratory chow and water were freely available.
Drugs: Lyophilized recombinant human IL-2 was obtained from Cetus Corp., Emeryville, CA. It has a specific activity of 3×10^6 Cetus Units/mg (8×10^6 BRMP Units/mg; 0.014 ng/ml endotoxin by

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standard limulus assay; purity 99%). It was reconstituted with sterile water, and diluted with 5% dextrose in water. The IL-2 vehicle (excipient) was used as a control. This is a buffer that does not contain IL-2, but has salts, bulking agent, and other excipient used in the IL-2 formulation. Dextrose in water (5%) and no treatment were used as two additional controls.

Antibodies: Commercially available antibodies were used to detect lymphocyte subsets by indirect immunofluorescence and included Lyt 5.2 (Cedarlane, Hornby, Ontario, Canada) for NK cells, MAB 1093 (Chemicon, Temecula, CA) for T-helper cells, MAB 1094 (Chemicon, El Segundo, CA) for T-suppressor cells, ACL-8952 mouse anti-mouse IgG (Accurate, Westbury, NY) for B-cells, and IGM-1603 goat anti-mouse IgG (Accurate, Westbury, NY).

Experiments: Study 1: Seventy-two mice were treated with a single i.p. dose of 10,000 or 75,000 Cetus Units of IL-2 or excipient at one circadian stage [5 hours after lights on (HALO); n = 3 per group]. Body weight changes were recorded and autopsies were performed at 24, 48, 72, 96, 120, 144, 168, and 216 hours after treatment with 10,000 Units and at 48, 72, 96 and 168 hours after treatment with 75,000 Units of IL-2 to determine spleen weight, wet and dry lung weight, total white blood count (WBC) and differential counts in spleen and peripheral blood. Study 2: Forty mice were treated with either 25,000 Units of IL-2, excipient, or 5% dextrose at 5 HALO on each of 3 consecutive days and compared to untreated controls (n = 5 per group). The same endpoints as above were studied 48 and 144 hours after the last injection. Study 3: Sixty mice were randomized to receive IL-2 at 25,000 Units/day over 3 days or an equal amount of excipient (control) i.p. at 1 of 6 circadian stages (each 4 hours apart). Single cell suspensions from spleen and bone marrow were prepared 48 hours after the last injection. Cell counts and lymphocyte subsets [total B-, T-helper, T-suppressor, and natural killer (NK) cells (Lyt-5.2+)] were determined in pooled samples from 5 animals per group.

RESULTS

In Studies 1 and 2, most responses to IL-2 were maximal 48 hours after completion of treatment and were dose- and schedule dependent. No consistent responses occurred after single low dose of IL-2, compared to no treatment, dextrose, or excipient. However, mean spleen weights (relative to body weight) increased by 25 and 43% after high dose given by single or divided injection, respectively (Fig. 1). The wet lung weight increased by 10% after a single high dose only. WBC in peripheral blood and spleen increased dose-dependently between 50 and 110% with peaks 2-3 days and 6-7 days. Differential counts showed a transient increase in granulocytes 48 hours after IL-2 treatment. In Study 3, significant endogenous circadian rhythmicity (>50% of daily mean) was present in control spleen weight (Fig. 2), the proportion of T-suppressor cells in the spleen (acrophase at 22 HALO; Cosinor p<0.04) and bone marrow (acrophase at 17 HALO; Cosinor p<0.03), and Lyt-5.2+ cells in the bone marrow (acrophase at 5 HALO; Cosinor p=0.06).
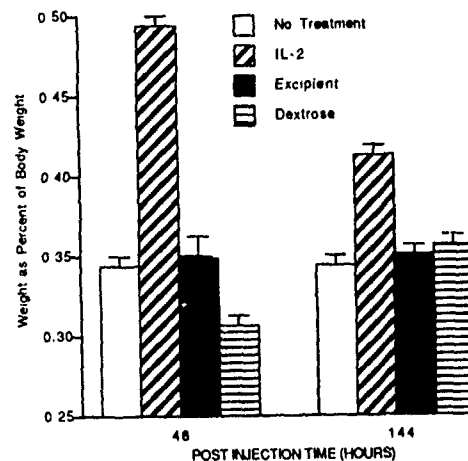

Fig 1. Median spleen weight 48 and 144 hours after no treatment, IL-2 (25,000 Unit/day x 3 days), excipient, or dextrose.

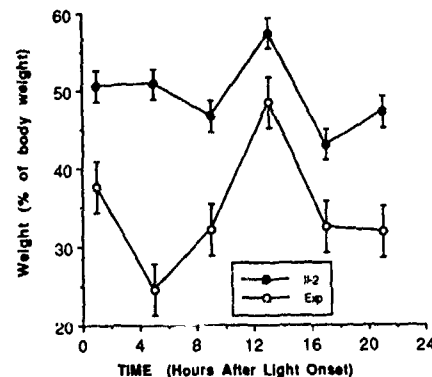Fig 2 Circadian variation of median spleen weight expressed as percent of body weight after IL-2 or excipient given at one of 6 circadian stages (ANOVA p<0.001, Cosinor p<0.05)

IL-2 increased the number of spleen cells by 16% overall, but up to 3-fold more when it was given during the activity span of the animals (acrophase at 17 HALO; Cosinor p<0.02) (Fig. 3). Spleen weight was correspondingly higher dependent upon IL-2 timing. The capacity of IL-2 to increase the splenocyte counts and modify the proportion of lymphocyte subsets was very different depending upon when in the day it was given (e.g., an increase of NK cells by >50% was only observed after treatment during animals' activity span) (Fig. 4-6). There was no significant increase in wet lung weight associated with IL-2 administration at a dose of 25,000 Units/day x 3 days; however, we noted an endogenous circadian rhythm in wet-dry lung weight in the control animals (acrophase at 9 HALO; double amplitude 80% around the mean; Cosinor p<0.01).

DISCUSSION

Our observation of endogenous circadian rhythmicity in immune cell numbers has also been reported by Levi et al. (1990), who found significant circadian rhythmicity and maximal NK-cell activity at the end of the activity phase in mice (acrophase at 24 HALO). In our studies, some of the biological effects after IL-2 administration in mice appear to be circadian stage dependent. The IL-2 treatment schedule used in Study 3 resulted in NK-cell induction only after drug administration during the activity phase of the animals. During other circadian stages, no or even negative responses occurred. The mechanism of the circadian organization of NK cells numbers and the response to IL-2 may be related to temporal changes in the regulation and expression of IL-2 receptors on NK cells (Kehrl et al., 1988). Toxicity and efficacy of other biological response modifiers including alpha-interferon (Levi et al., 1990), tumor necrosis factor (Langevin et al., 1987), and the plasma levels and effects of the growth factor erythropoietin also differ substantially and reproducibly depending upon the circadian stage (Wide et al., 1989; Wood et al., 1990). Therefore, time of day of biological response modifier administration, including IL-2, should be considered for study designs and interpretation of immunological investigations.
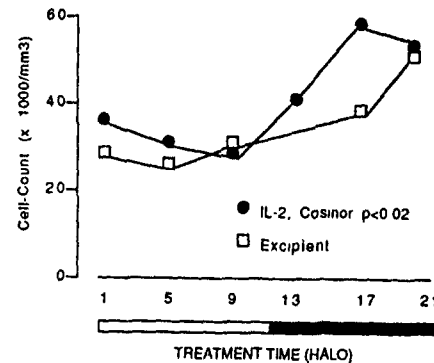

Fig. 3. Circadian variation in the number of spleen cells after treatment with IL-2 or excipient
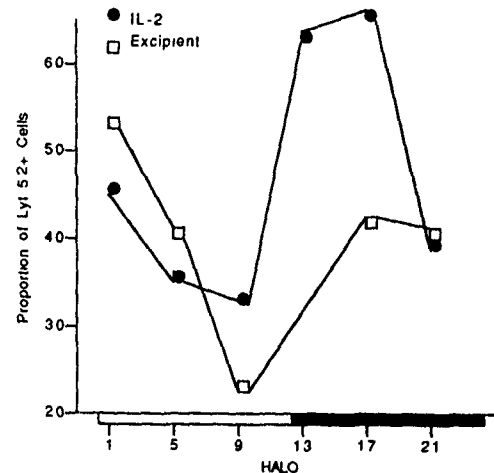

Fig. 4 Circadian variation in the proportion of spleen Lyt 5 2+ cells after IL-2 or excipient given at different circadian stages
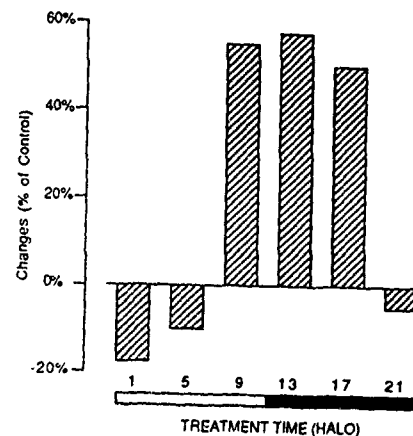

Fig 5 Relative changes after IL-2 in the proportion of spleen Lyt 5 2+ cells (percent of excipient controls).
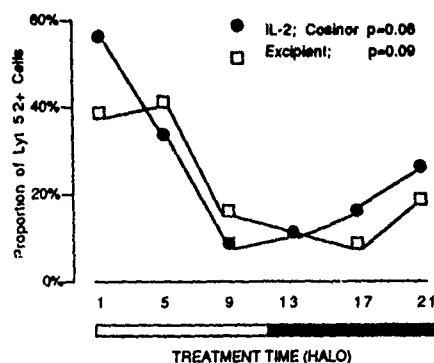
Fig 6 Circadian variation in the proportion of Lyt+
cells in the bone marrow after IL-2 or excipient.

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HOW TO ACCURATELY EVALUATE GROWTH FRACTION POTENTIAL DOUBLING TIME AND CELL LOSS IN ANIMAL AND HUMAN TUMOR SYSTEMS WITH CIRCADIAN KINETICS

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ABSTRACT

The author reviews the bias in cell kinetics computation of animal or human systems submitted to synchronous waves of proliferation. Classical methods postulating asynchronous cell division activity are no longer of value in such systems. By repeating measurements of labelling indices and of determination of cell cycle time parameters, it became possible to integrate 24 hrs waves of division activity. It allowed to obtain a better estimate of internal potential doubling times. Several examples of the methodology proposed are presented.

KEY WORDS

Cell kinetics - Circadian rhythms.

INTRODUCTION

Classical methods for evaluation of cell kinetics in proliferative systems (tumoral or not tumoral ones) postulate asynchronous proliferation. However circadian rhythms of division have been established in animal and human systems actively dividing, ie, normal tissues, such as bone marrow, GI tract, cornea, epidermis... and also tumor tissues (experimental: methylcholantrene induced sarcomas, hamster islet cell tumor, lewis lung carcinoma, breast carcinoma; human : ovarian cancer, epidermoid carcinoma..). For an accurate determination of internal kinetics in such systems, one has to question about value of classical methods. We will review here a few examples of appropriate and inappropriate methods for precise determination of cell proliferation activity in circadian systems.

Classical methodology

The potential doubling time (Tdpot) represents the time necessary
to double the cell population in a system actively dividing .
Several measurements have been proposed to obtain this evaluation
especially stathmokinetic methodology (measure of the rate of
mitotic accumulation after mitotic blockade obtained from a
fusorial poison (ie, colchicine, colcemid, vinblastine,...) and
estimation from growth fraction (GF) and cell cycle time (Tc) ÷

$$Tdpot = \ln 2 / \ln (1 + GF) . Tc \quad (A)$$

The GF evaluation can be devised from
(1) the Mendelssohn technic (ratio between labeling index - LI and
percent labelled mitosis -PLM at 120 hours after a tritiated
thymidine 3HTdR injection, or from
(2) the ratio ÷ $GF = \dfrac{TC}{TS} . LI$ (B)

where TC & TS represent the duration of cell cycle and S phase and
LI the percentage of cells labelled after a H3TdR injection (ie
cells engaged into the DNA synthesis).
Cell cycle and S phase durations can be estimated from analysis of
the PLM curve drawn after a 3HTdR injection .
The difference between actual doubling time (TD) and Tdpot
represent the cell loss ;

$$O = 1 - \dfrac{Tdpot}{TD} \quad (C)$$

Evaluation of classical methods in experimental tumor system with
circadian division activity. Proposition of a new methodology.

We have shown that most of the methods described below are no
longer appliable in circadian systems especially methods
postulating asynchronous proliferation . As an example, the
stathmokinetic method is inoperative as, according to the hour of
the mitotic blockade agent administration, the rate of mitotic
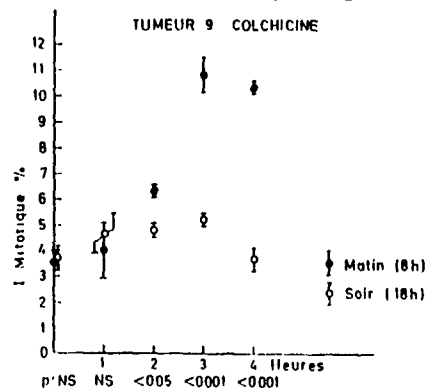accumulation varied substantially (fig. 1).

Fig 1: Mitotic accumulation in mouse sarcoma T9 after colchicine
        administration (1 mg/Kg) at 8 am and at 6 pm (day 8 of growth.

Similarly, the Mendelssohn technic postulating a desynchronisation
of PLM wave obtained after 3-5 division periods ( ) gave completely
aberrant results in such systems (Table I).

Table I: Mean values of LI (%), PLM (%) and GF (LI/PLM) obtained 120 hours after a tritiated thymidine injection (1 uCi/gr) performed at 12 am and at 12 pm at days 8,15,22,29 in T9 and T10 mouse sarcomas

	Injection 3HTdR	12 h				24 h	
		LI	PLM	GF	LI	PLM	GF
J8	T 9	4 02	2 33	1 72	3 31	3 00	1 10
	T 10	3 10	1 66	1 87	2 48	1 66	1 49
J15	T 9	3 51	2 30	1 53	1 73	2 30	0 75
	T 10	3 33	2 00	1 67	2 17	3 30	0 66
J22	T 9	2 71	2 00	1 36	2 72	3 50	0 78
	T 10	1 25	0 66	1 89	1 09	1 33	0 82
J29	T 9	4 15	3 50	1 19	1 57	2 00	0 70
	T 10	2 35	2 00	1 18	1 27	1 33	0 95

By repeating the PLM curves at various diurnal times (Fig. 2a + b), one can evidenced substantial variability of all the cell cycle phases along the diurnal period .
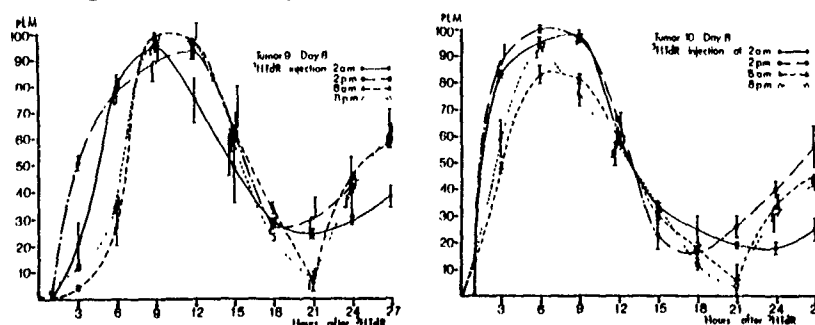
Fig 2: Percent labeled mitosis curves in mouse sarcomas T9 and T10 after tritiated thymidine injections (1 uCurie/gr) at 2 am, 8 am, 2 pm, 8 pm (day 8 of growth.

However, repetition of those PLM evaluations allowed calculation of a 24 hrs mean duration of cell cycle (TC-1) and S phase (TS-2). Similarly by determining the circadian waves of proliferation indices, one can evaluate the 24 hrs mean mitotic (MI) or labelling (AI-3) indices. Therefore knowing (1) + (2) + (3) one can calculate a 24 hrs-mean value for growth fraction (equation B) and furthermore of 24 hrs-mean Tdpot (equation A) and 24 hrs-mean cell loss (equation C). Similar results have been obtained from the graphical integration of a 24 hrs LI wave taking into account mean TS duration. Furthermore these results were perfectly consistent with those obtained from a 24 hrs continuous H3tdR labelling allowing direct measurement of cells passing through the DNA synthesis during the period of exposure. The perfect concordance between direct measurements (continuous labelling) with proposed methodology confirmed the validity of the latter (Table II). The table II gives 2 examples of those computations in 2 MCA sarcomas of mice and in a hamster pancreatic islet cell tumor.

C. Focan

Table II: Cell kinetics computations in circadian systems

Mean 24 hrs	Mouse sarcoma T9	Mouse sarcoma T 10	Hamster islet cell tumor
MI (%)	3 7+/-0 5	2 7+/-0 3	0 95+/-0 1
LI (%)	34 8+/-7 9	33 4+/-4 4	17 3 +/-2 3
Tc (hrs)	20 5	28 5	31 6
TS (hrs)	10 2	10 4	16 7
GF	0 7	0 84	0 33
Cells in DNA synthesis(%)			
(1)	90 3+/-1 1	78 9+/-3 5	---
(2)	88 3	79 2	26 1
Tdpot (hrs)			
(3)	26 3	30 4	77 2
(4)	26 6	30 1	92.0
TD (hrs)	134	67	278
0 (3)	0 8	0 55	0 67
(4)	0 8	0 55	0 72

(1)continuous labelling, (2)LI wave integration (3)from GF,
(4)from continuous labelling and/or graphical integration

Extrapolation of results to human circadian tumor system

Extrapolation to human system could become possible from the
computation of (1) 24 hrs 'LI wave and 24 hrs mean LI and (2) the
knowledge of median values of TC and TS in human solid tumors. The
24 hrs LI wave can be obtained by repeated mesurements of LI alone
a 24 hr period . We have established median duration of TC and TS
from 30 PLM determinations in human solid tumors (probit log
distribution; Fig 3).
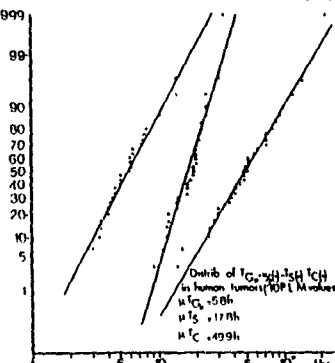
**Fig 3. Distribution of cell cycle parameters from PLM curves
performed in 30 human solid tumors.** Median values were for Tc: 49.9
hrs, for TS: 17.8 hrs and for G2: 5.8 hrs.
From those computations, one can further evaluate GF, Tdpot and
cell loss, if we have relevant informations on the "clinical"
doubling time of the evaluated tumor.

DISCUSSION - CONCLUSION

From examples presented below, we can conclude that classical
computations proposed for evaluating cell kinetics in proliferative
systems submitted to circadian division rhythmicity are obsolete.
However, from the determination of mean (or mesor) 24 hrs kinetic
parameters, precise evaluation of internal kinetic (ie, Tdpot) can
be drawn. We have shown that these calculations fitted perfectly
with direct measurements, ie continuous H3TdR continuous labelling
over 24 hrs. The possible extrapolation to human solid tumor
systems (from evaluation of clinical doubling times and mean 24 hrs
labelling indices) could render cell kinetics operational as real
tumor markers for guiding circadian timed chemo- and/or
radiotherapy. The recent application of cell cytofluorometric
methodology on repeated punctures of accessible tumors or of
immunohistochemical staining methods on true cut biopsies or cell
suspensions could render kinetic computation more easily handled.
PRINCIPAL REFERENCE: Focan C.,PhD Thesis, Liège, 1985, pp 252.

CIRCADIAN TOLERANCE TO ETOPOSIDE AND CISPLATIN AS FIRST LINE TREATMENT FOR ADVANCED LUNG CARCINOMA

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ABSTRACT

The authors reported on the clinical toxicity of a randomized
chemotherapy trial administering etoposide (100 mg/m2, days 1
+ 2 + 3) given either at 6 am (A) or at 6 pm (B) and
cisplatin 100 mg/m2 at 6 pm on day 4. This chemotherapy
protocol was administered to previously untreated patients
suffering from an advanced lung cancer.
Despite some differences among both groups (less severe
nausea-vomiting, less ototoxicity and less dose reductions in
group B, but more severe alopecia in this group - p < 0.006
-0.045), no difference in overall dose intensities could be
detected with regard to the time scheduled chemotherapy.

KEY WORDS

Etoposide - Cisplatin - Lung Cancer - Chronotherapy.

INTRODUCTION

Chronotolerance has been reported in rodents and in human
beings for the administration of both etoposide and cisplatin
(1-3). The association of those drugs seemed to remain one
of the most efficient recommended for treating non small cell
lung carcinoma, as well as small cell lung carcinoma (4,5).
In a previous chronotherapy trial administered to advanced
solid tumors (mainly advanced lung cancers), including a
sequential 40 hours chemotherapy with methotrexate,
vinblastine and cyclophosphamide, beginning either at 10 am
or at 10 pm, we were able to evidence a difference both for
response and survival rates in favour of the group treated
from 10 am, (6). We propose to analyse the tolerance of a

sequential chemotherapy associating etoposide and cisplatin
given to previously untreated patients suffering from
advanced lung carcinoma .

MATERIAL AND METHODS

The following conditions were required for inclusion :
histological proof of a lung carcinoma; at least stage III
disease according to the AJC criteria, measurable or
evaluable disease; no prior chemo- or radiation therapy, no
CNS symptomatic involvement; no concomitant corticotherapy;
age < 75; sufficient clinical indices (ECOG <= 2; Karnofsky
>= 50) and lack of failure of bone narrow, kidney or liver
functions
After stratification according to histology (NSC; SC) and
stage (limited/extensive) and after informed consent,
patients were randomized to receive etoposide 100 mg/m2
given at days 1, 2, 3 either at 6 am (A) or at 6 pm (B) and
cisplatin 100 mg/m2 at day 4 at its best timed schedule, ie
6 pm (3). No dose intensification was intended.
The evaluation of toxicity was performed according to the WHO
grading (7), and was refered either to the course numbers or
to the maximal toxicity per patient The dose intensity was
expressed as recently recommended, ie, in mg/m2/week (8); the
ratio between the dose effectively given versus the dose
projected (ie, for etoposide 100 and for cisplatin 33.33
mg/m2/week) was also established. The student's t and chi
Square tests were used when appropriate.

RESULTS

Patients characteristics (table I)

At present (02-19-90), 141 patients (A > 65; B > 76)
fulfilling inclusion criteria have been randomized in the
trial. They were well balanced with regard to prognostic
characteristics (table I)   They received a mean number of 3
courses. 112 cases (A > 54; B > 58) and 356 courses (A :
169; B > 187) were fully evaluable for the analysis of
toxicity.

Toxicity (table II)

The general toxicities are presented in table II. No
difference in hematological toxicity was evidenced at this
cut-off analysis on the contrary to what has been reported
earlier (9). It must however be pointed out that the
hematological tolerance to the protocol was rather mild as
WHO grades III-IV for WBC toxicity were encountered only in
7 % of courses in group A and 11 % of courses in group B.
We found less severe nausea-vomiting and less ototoxicity in
group B but more severe alopecia in this group. No
difference among both groups was encountered with regard to
the evaluation of the maximal toxicity per patient, except
for ototoxicity (p < 0.043 in favour of group B). Other
toxicities were reported in 20 cases in group A and 17 cases
in group B, let us point out supraventricular arrythmias (A >
4; B > 1) and benign diarrhea (A > 6; B . 5).

TABLE I -CHARACTERISTICS OF RANDOMIZED PATIENTS

	Whole group (141)	Group A (65)	Group B (76)
MEAN AGE	63	61	64
(range)	(39-80)	(39-80)	(39-80)
SEX M	116	54	62
F	16	8	8
Unknown	9	3	6
HISTOLOGY			
NSCLC Total	112	47	65
Epidermoid	61	24	37
Adenoc	34	15	19
Anaplasic	12	4	8
Mixed	2	2	0
Unspecified	3	2	1
SCLC	29	18	11
STAGE Limited	49	26	23
Extensive	71	30	41
Unknown	21	9	12
EVALUABLE	112	54	58
FOR TOXICITY			
MEAN NB OF COURSES	3 1±1 6	3 1±1 7	3 2±1 6
(range)	(1-7)	(1-7)	(1-6)
EVALUABLE COURSES	356	169	187

TABLE II-GENERAL TOXICITY - WHO GRADES [Nb - (%)]

Courses analysed: 356

	A (169)	B (187)	P
HB>=2	20 (11 8)	34 (18 2)	ns
WBC 3+4	12 (7 1)	20 (10 7)	ns
Granulo 3+4	37 (21 9)	26 (13 9)	ns
Platelets 3+4	5 (2 9)	5 (2 7)	ns
Nausea-vomiting >=2	104 (61 5)	87 (46 5)	0 005
Alopecia >=2	86 (50 9)	112 (59 9)	0 045
Infections >0	12 (7 1)	14 (7 5)	ns
Kidney >0	10 (5.9)	10 (5.3)	ns
Stomatitis >0	0 (0)	1 (0 05)	ns
Neurology >0	11 (6 5)	6 (3 2)	ns
Ototoxicity>0	14 (8 3)	1 (0 05)	0 001

Dose reports, - reductions and - intensities

No difference between groups A and B with regard to dose reports (A : 20.1 %; B : 19.3 %) could be evidenced but a difference emerged in favour of group B for dose reductions rate of etoposide (A : 24.3 %, B : 15 % - p < 0.02) as well as of cisplatin (A : 13 %; B : 4.8 % - p < 0 006). This difference was not translated in overall dose intensities - DI [DI mg/m2/week - ratio dose given versus dose projected: (1) for etoposide : A : 0.81 +/- 0.16; B : 0.84 +/-0.15; (2) for cisplatin . A : 0 84 +/- 0.15, B . 0.86 +/-0.13). Similarly no difference appeared when the same analysis was performed according to the number of courses received (1,2,3,4, >= 5)

CONCLUSIONS - COMMENTS

In this report, we were unable to confirm previous assessments regarding the hematological chronotoxicity of etoposide(2,9). According to Krakowski's evaluation, this drug was estimated to be best tolerated when administered around 6 to 7 am in the morning (2); this study included however patients previously treated either by chemo and/or

radiotherapy, which was not the case in our actual trial.
Our first presentation of analysed courses concluded also in
the same direction (9); this was not confirmed in the present
analysis, but as pointed out, hematological toxicity in the
present study appeared to be easily manageable. The marginal
differences in tolerance evidenced between groups A and B (ie
less nausea-vomiting and ototoxicity but more severe alopecia
in group B), were not translated into overall doses of
chemotherapy effectively given; indeed strictly no difference
in overall dose intensities could be recorded. In
conclusion, at the present time, we found no significant
chronotolerance of a sequential chemotherapy associating
etoposide and cisplatin given to patients previously
untreated, suffering from an advanced lung carcinoma.
Despite those negative results, according to the initial
statistical hypothesis, precluding a difference of 20 % in
favour of group A, definitive conclusion will only be
available after inclusion and evaluation of at least 166
patients. Therefore the study is going on; it could be hoped
that a difference will ermerged among both groups regarding
response and survival rates ?

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CIRCADIAN RHYTHMS IN EXPRESSION OF CELL SURFACE ANTIGENS OF MURINE SPLEEN CELLS

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ABSTRACT

The circadian rhythms of the major classes of splenic lymphocytes (T, T helper, T suppressor, and B) and that of circulating corticosterone and estradiol in the C3HeB/FeJ mouse were measured. Correlations among these parameters were also defined.

KEY WORDS

Circadian, Lymphocyte Surface Antigens, Corticosterone, Estradiol

INTRODUCTION

Physiologic functions reveal marked cycles of varying length (Halberg, 1960). Ratajczak et al. (1988) showed the importance of appropriate timing within the estrous cycle for the removal of a primary mammary adenocarcinoma in prevention of subsequent metastasis in the C3HeB/FeJ mouse. The present study investigated the normal C3HeB/FeJ mouse to further elucidate hormonal effects on the lymphocyte surface antigens, which are known to define the function of the cells.

MATERIALS AND METHODS

Chronobiology Boxes were prepared as per Dr. L.E. Scheving (personal communication). Three boxes were placed in two rooms which were on opposite 12:12 hour light:dark cycles. Each box had a timer to control its interior light to a 12:12 hour light schedule. Time is referred to as hours after lights on (HALO). The onset of light was staggered by four hours between boxes.

Mice (C3HeB/FeJ, 3-5 weeks old) were purchased from Jackson Laboratories. Male mice, two per cage, were in close proximity to the females, four per cage. All animals were fed laboratory chow and tap water ad libitum. One half of the female experimental groups were killed by exsanguination on day 27, the remainder, day 28. A total of 72 female mice were tested, 12 from each time point. Mice were anesthetized with carbon dioxide and bled via the retroorbital plexus. Sera were stored at -70°C.

Lymphocyte Surface Antigen Determination: Spleens were aseptically removed, and single cell suspensions were prepared in MEM (Gibco, Grand Island, NY) #33C-1430, pH 7.6, plus 0.1% NaN3. Lymphocyte cell surface antigens were identified by flow cytometry utilizing an Epics V Fluorescent Activated Cell Sorter (FACS). Cells were stained with antibodies conjugated with fluorescein isothiocyanate: monoclonal antibodies to Thy 1.2 (total T cells, T_m, Becton Dickenson), Lyt 2 (T suppressor cells, T_s, Becton Dickenson), L3T4 (T helper cells, T_H, Sera Labs), and polyclonal F(ab)'2 goat anti-mouse immunoglobulins (B cells, B, Zymed) were used to determine lymphocyte subtypes. Propidium iodide negativity determined cell viability and only viable cells were analyzed.

Hormones were quantitated by radioimmunoassay (Radioassay Lab, ICN Biomedical, Inc.)

Statistics: The least-squares fit of a cosine curve (Nelson, 1979) was used to determine rhythmicity of cell surface antigen expression and hormone levels in peripheral blood.

RESULTS

Fig. 1

Prior to independent analysis, each parameters' data was transformed into a percent of mean. Circadian rhythms have been demonstrated by rejection of the 'no rhythm assumption' in the context of the single cosinor method for the following immunologic variables: percent of cells expressing the cell surface antigens Thy 1.2, L3T4, Lyt2, and immunoglobulin (Fig. 1). Circadian variation was also described for blood corticosterone in which the p value for the test of 'no rhythm assumption' was <0.05 when the mean values of each time point were examined. The acrophase of the circulating corticosterone occurred just prior to the acrophase of the immunologic variables. Estradiol data exhibited a change of borderline statistical significance (p=0.072), acrophase -342°.

Circadian Rhythm in Circulating Corticosterone and Immunologic Variables
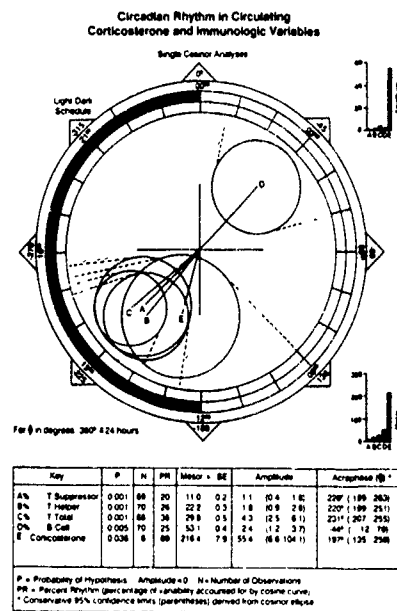
TABLE 1 - Circadian Values for Hormones and Cell Surface Antigens

	0 HALO	4 HALO	8 HALO	12 HALO	16 HALO	20 HALO
Corticosterone (ng/ml)	145.7 ± 35.5	180.9 ± 32.6	243.3 ± 49.1	249.6 ± 60.0	263.6 ± 75.0	215.5 ± 48.5
Estradiol (pg/ml)	24.9 ± 1.9	25.1 ± 2.4	21.8 ± 2.2	20.4 ± 0.9	22.8 ± 2.7	27.2 ± 2.8
% THY1.2	26.8 ± 0.8	26.4 ± 0.7	27.0 ± 1.0	34.0 ± 1.0	32.9 ± 1.6	31.9 ± 1.5
% L3T4	20.7 ± 0.7	20.6 ± 0.5	21.3 ± 0.6	24.5 ± 0.7	23.0 ± 0.8	23.0 ± 0.7
% LYT2	9.7 ± 0.4	10.3 ± 0.3	10.4 ± 0.5	12.1 ± 0.4	11.5 ± 0.6	11.8 ± 0.5
% IgG	54.5 ± 0.6	54.4 ± 0.9	54.8 ± 0.5	51.1 ± 0.6	49.7 ± 1.4	53.8 ± 0.8
Cell Number x 10⁵	1329 ± 234	1257 ± 149	1004 ± 68	1141 ± 65	1318 ± 145	1186 ± 72
Abs Number x 10⁵ THY1.2	362 ± 61	294 ± 17	300 ± 20	391 ± 28	307 ± 40	
Abs Number x 10⁵ L3T4	270 ± 41	231 ± 15	212 ± 14	277 ± 12	297 ± 47	276 ± 23
Abs Number x 10⁵ LYT2	130 ± 23	118 ± 7	102 ± 6	137 ± 7	147 ± 13	162 ± 14
Abs Number x 10⁵ IgG	737 ± 144	611 ± 40	550 ± 38	584 ± 36	666 ± 29	633 ± 34

Circadian values for hormones and cell surface antigens are listed in Table 1. Using all the data, correlations were tested among the percent of cells expressing antigen, the absolute number of cells expressing antigen, and the levels of hormones. Among the percents of the various T antigens, r's range from 0.65 to 0.76 (p<0.0001) and among absolute numbers of T cells r's range from 0.91 to 0.94 (p<0.001). Among the percent of T antigens and the percent of B, r's ranged from 0.29 to 0.50 (p<0.02 to 0.001). Correlations tested between percent of T and percent of B were negative: r=-0.39 to -0.64 (p<0.001 to 0.0001). In contrast, highly positive correlations were found between absolute numbers of T and B: r=0.72 to 0.89 (p<0.0001). Cellularity varied, and could account for the shift from negative correlations for the percents to positive correlations for the absolute numbers. Circulating corticosterone showed a weak negative correlation with absolute numbers of B, percent of B, and estradiol: r=-0.30 (p=0.02). r=-0.22 (p=0.07), and r=-0.28 (p=0.02) respectively.

When analyzed at the six time points separately, correlations computed between corticosterone and absolute numbers of lymphocyte subsets changed systematically from positive at 4 HALO to negative at 12 and 16 HALO (Fig. 2). The correlations for B, T_S and T_H were statistically significant only at one circadian stage, 16 HALO. Estradiol levels correlated with absolute numbers of B at 12 HALO (Fig. 3). As expected, strong correlations (p<0.05) were observed among each of the comparisons of T, T_H, T_S, and B (Fig. 4).
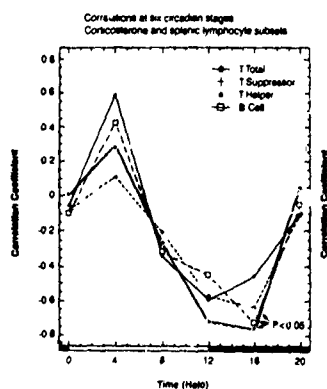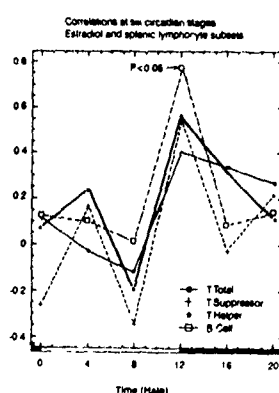
Fig. 2   Fig. 3   Fig. 4
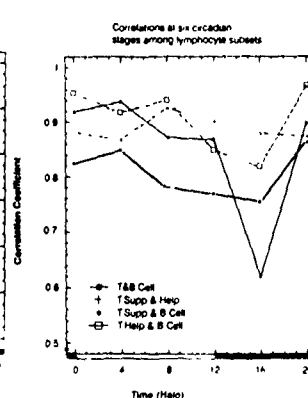
DISCUSSION

The present study revealed strong circadian rhythms and relative time courses for splenic cell surface antigens and corticosterone levels in circulating blood of the C3HeB/FeJ mouse. These findings support those of many investigators in the human, including Levi (1985) who showed circadian rhythm for circulating lymphocytes, and Weitzman (1974) who studied several hormones including cortisol. In the mouse, B and T cells from the spleens as well as corticosterone levels were shown to display marked circadian rhythms (Kawate, 1981). In general, our data correspond with theirs, despite the fact that Kawate used two different methods for determining B and T, cell surface fluorescence and cytotoxicity, and we used the same method, FACS analysis for all antigens. In addition we identified T cell subsets, T_H and T_S, and estradiol

levels. It is interesting that in the current study percent of
cell subtypes were correlated negatively but absolute numbers were
correlated positively. The total number of cells per spleen varied
greatly (from 709x10^5 to 3775x10^5). A circadian rhythm was not
revealed in the cellularity, however it could explain the switch
from negative to positive correlations. The cellularity is
multiplied by the percent to derive absolute number. As the spleen
increased in cellularity, all lymphocyte subsets would increase in
absolute number, resulting in positive correlations. This study
reflects a portion of a larger investigation concerning the estrous
cycle and its influence on cell surface antigen expression. It is
expected that the estrous cycle affected estradiol levels to a
greater extent than did circadian rhythm. In addition,
dehydroepiandrosterone was measured in the brains of these animals,
and levels are being correlated with parameters examined in this
study. It is anticipated that a new understanding will be apparent
when interactions, "feedsidewards", of these three hormones'
effects will be assessed (Kanabrocki et al., 1983 and Jo et al., in
press).

It is interesting that estradiol correlated with absolute numbers
of B at 12 HALO. The significance of this correlation remains
unknown. The correlation of corticosterone levels with cell
surface antigens (T$_H$, T$_S$, and B) at 16 HALO is in keeping with the
endogenous rhythm of corticosterone which peaks just prior to that
time. See Fig. 1. These data cannot imply that corticosterone or
estradiol cause cell surface antigens to be expressed. A study is
in progress in which adrenals or ovaries were removed and hormones
reconstituted exogenously to prove the influence of these hormones
on expression of these cell surface antigens.

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THE ROLE OF DIHYDROPYRIMIDINE DEHYDROGENASE (DPD) AND THYMIDINE PHOSPHORYLASE (dThdPase) IN THE CIRCADIAN VARIATION OF PLASMA DRUG LEVELS OF 5-FLUOROURACIL (FUra) AND 5-FLUORODEOXYURIDINE (FdUrd) FOLLOWING INFUSION OF FUra OR FdUrd

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KEYWORDS

INTRODUCTION

For the past three decades the fluoropyrimidines, 5-fluorouracil (FUra) and its nucleoside 5-fluoro-2'-deoxyuridine (FdUrd) have been utilized in the treatment of cancer. FUra, the pyrimidine base analog of uracil, is used primarily in the management of colorectal, breast, and ovarian cancer. In contrast, FdUrd is utilized in the management of hepatic metastases of advanced gastrointestinal adenocarcinoma (Ensminger et al., 1978) and, more recently, in systemic treatment of renal cell carcinoma (Hrushesky et al., 1988). Following their administration, these drugs are anabolized to their active metabolites resulting in either suppression of DNA synthesis, or interference with DNA and/or RNA functions. Dose-limiting toxicity of both FUra and FdUrd have been reported (Diasio and Harris, 1989).

In an attempt to improve the therapeutic index of these fluoropyrimidines continuous infusion regimens have been developed and studied (Hrushesky, et al., 1988). With the advent of ambulatory drug delivery pumps, continuous infusion of these drugs has become more clinically feasible. Recent studies have suggested that the therapeutic effectiveness of FdUrd could be further optimized if it is continuously infused in a "time-modified" schedule. One such schedule which has been demonstrated to have increased therapeutic effectiveness delivers 68% of daily dose during the six-hour segment between 3:00 PM and 9:00 PM while the remaining 32% is delivered over 3 successive, six-hour segments. (Roemeling and Hrushesky, 1989). The basis for this improved therapeutic index remains unclear. Additional studies with continuous FUra infusion have also observed a circadian variation in plasma FUra concentration (Petit et al., 1988; Fujii et al., 1989; Harris et al., 1990). These findings led our laboratory to question whether the biochemical basis for the improved therapeutic index and the variation in plasma FUra level is perhaps due to a variation in key enzymes of pyrimidine metabolism. Since approximately 80% of administered FUra is rapidly catabolized, it is important to examine the circadian variation of catabolic enzymes, particularly if the rhythm of such enzymes can explain the reported variability in plasma drug level.

Dihydropyrimidine dehydrogenase (DPD), the initial enzyme in FUra catabolism, is a major factor determining the availability of FUra. Since "time-modified" programming of drug administration has been demonstrated to improve the therapeutic index of patients treated with FdUrd, we examined the activity of thymidine phosphorylase (dThdPase), the major enzyme in the breakdown of FdUrd, to determine if it has a circadian pattern and to evaluate its relationship to the circadian variation of DPD activity that we previously demonstrated (Harris, et al., 1990).

METHOD

Patient Characteristics and Blood Collection

All patients were admitted to the General Clinical Research Center (GCRC) at the University of Alabama at Birmingham on an institutionally approved protocol. All patients received continuous infusion of FdUrd (0.15 mg/kg/day) for 7 days. The patients had typical sleep and activity patterns (onset of sleep, 9 - 11 p.m.; awakened 6 - 7 a.m.). Four patients (2 men, 2 women) participated in this study. The age of the patients ranged from 52 to 80 years and all four patients had biopsy-proven renal cell carcinoma. Following admission to the GCRC at 8:00 AM, a heparin-lock was placed in the forearm in order that blood samples could be drawn with minimal discomfort to the patient during sampling. Blood (25 ml) was collected into a 30-ml syringe containing 5 ml of heparin (1000 units/ml) starting at 9:00 AM and continuing at three hours intervals until 6:00 AM the following day.

Assay of dThdPase and Statistical Analysis. Immediately after its collection, the blood was added to ¹5 ml of Hank's balanced salt solution in a 50-ml centrifuge tube. Plasma was removed by centrifugation. The cells were then resuspended and separated with Histopaque (Sigma Co., St. Louis, MO) as previously described (Diasio et al., 1988). The peripheral blood mononuclear cell fraction was removed and sonicated five times for periods of 10 sec in ice cold 135 mM sodium phosphate buffer (pH 7.5).

The cell preparation was centrifuged (20,000 × g for 3 min) and dThdPase activity was assayed as previously described (Chu et al., 1984). Briefly, the supernatant was incubated at 37°C in a mixture containing 20 mM potassium phosphate (pH 8.0), 1 mM EDTA, 1 mM β-mercaptoethanol, and 1000 μM [6-³H]FdUrd (0.35 μCi/μMol). The reaction was stopped by removing an aliquot (300 μl) at a designated time and adding it to an equal volume of ice-cold 100% ethanol. ³H-FdUrd and ³H-FUra were quantitated by reverse-phase HPLC as described earlier (Sommadossi et al., 1982). For each time period, enzyme activity was analyzed from the slope of four time points over the initial 20 min during which the reaction was linear. Enzymatic activity was expressed as nanomoles of total ³H-FUra formed per minute per 10⁶ cells.

The time series data obtained over the 24 hr period were analyzed by the "Cosinor" analysis method as described earlier (Halberg et al., 1972). In an attempt to account for interpatient variability of observed values for dThdPase activity, the data was normalized by expressing each value as a percentage of the 24-hr mean for that patient. The data was fitted to a cosine wave by regression analysis utilizing least squares (Reinberg and Smolensky, 1983). Using this analysis three parameters were quantitated. These include the amplitude (i.e., maximum or minimum value from the mean), the mesor (i.e., the rhythm-adjusted mean), and the acrophase (i.e., time of maximum or minimum value)

RESULTS

As determined by cosinor analysis, dThdPase activity did not exhibit a circadian pattern. Figure 1 shows the overall (average of 4 patients) percent 24-hr mean activity of dThdPase to be random and not circadian. Table 1 shows the cosinor analysis obtained for each individual patient. The range of dThdPase 24-hr mean activity is 24-36 nmol/min/10⁶ cells.

Table 1. Summary of dThdPase activity in peripheral blood mononuclear cells in patients receiving FdUrd by continuous infusion (0.15 mg/kg/day)§.

Patient #	Mesor* ± SD	Maximum‡	Time of Max§ (hr)	Minimum‡	Time of Min§ (hr)	R^2	P
1	26.74 ± 2.18	28.23	3.6	25.24	15.5	0.27	0.46
2	35.57 ± 5.88	40.45	10.9	30.69	23.0	0.39	0.29
3	25.54 ± 2.58	26.75	2.0	24.33	13.9	0.13	0.71
4	24.13 ± 3.02	26.77	14.9	21.49	27.0	0.44	0.24

§ Activity is expressed as nmoles/min/10⁶ cells
* Mesor = rhythm-adjusted mean
‡ Maximum/minimum = mesor ± amplitude (not shown)
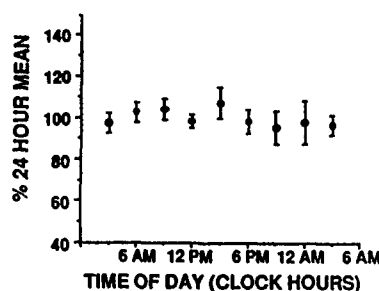§ Time is expressed on a 24-hour scale

Fig. 1. Overall variation of dThdPase activity in peripheral blood mononuclear cells in 4 patients receiving FdUrd by continuous infusion (0.15 mg/kg/day). Values represent mean ± SE.

As reflected by the R^2, the best fit of the data to the cosinor wave was very low and the statistical variation between its peak and trough was not significant. In order to compare the activity of dThdPase to that of DPD in peripheral blood mononuclear cells, we re-evaluated our data from an earlier study (Harris, et al., 1990) and re-expressed DPD activity as nmole/min/10^6 cells. As shown in Fig. 2, the mean specific activity of dThdPase for each individual patient was significantly higher than the mean specific activity of DPD obtained from seven previous patients.
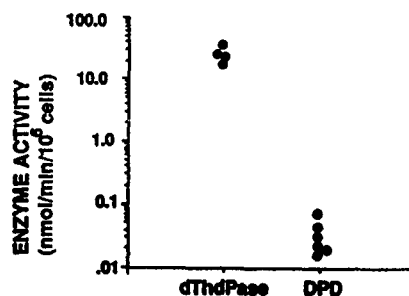
Fig. 2. 24-hr mean specific activity of dThdPase and DPD in individual patients.

DISCUSSION

In an attempt to determine the biochemical mechanism for the improved therapeutic index, we examined the catabolism of FdUrd in patients receiving continuous FdUrd infusion. In the present study no significant circadian variation of dThdPase activity in peripheral blood mononuclear cells was observed. We were not able to correlate the plasma concentration of FdUrd with the corresponding activity of dThdPase due to the very low level of FdUrd present in the plasma of the patients (infusion rate = 0.00625 mg/kg/hr). While we were not able to assess the FdUrd plasma level, it is interesting to note the result of a recent study with 5-iodo-2'-deoxyurdine (IdUrd) (Klecker et al., 1985), another substrate of dThdPase. In their study, Klecker et al (1985), infused IdUrd into patients at a rate as high as 1200 mg/m^2/12 hrs, demonstrating that the primary metabolite 5-iodouracil (IUra) did not reach a steady state during therapy. Furthermore, at the end of the infusion, the maximum plasma IUra concentration was 100 μM, approximately 10 fold higher than the simultaneous IdUrd plasma concentration. Though the purpose of the study was not to examine the circadian variation of dThdPase, this data would suggest that FdUrd plasma level would not have a circadian variation.

While dThdPase does not have a circadian variation, time-modified programming may still be based on circadian variation of other important pyrimidine catabolic steps. Since the FUra formed from the catabolism of FdUrd would be further catabolized by DPD, an enzyme that we have already shown to follow a circadian rhythm, we attempted to compare the specific activity of dThdPase to that of DPD. As shown in Fig. 2, the 24-hr mean activity of dThdPase for each patient was observed to be substantially higher than that of DPD activity. This is in agreement with a previous study (Naguib et al., 1987) which demonstrated that dThdPase activity was greater than DPD activity in human lymphocytes. This suggests that of these two catabolic steps, DPD is rate-limiting in the degradation of FdUrd.

In summary, this study suggests that if the reported chronotoxicity of FdUrd is due to a variation in the fluoropyrimidine (FdUrd or FUra) plasma level, then this variation may be regulated by DPD and not dThdPase. Another conceivable enzyme responsible for the chronotoxicity of FdUrd might be uridine phosphorylase. Circadian variation in the catalytic activity of this enzyme has been shown in mouse liver (el Kouni et al., 1989). More work is needed in order to investigate the molecular regulatory control of these enzymes and the possible circadian activity of other enzymes (i.e., anabolic) which can potentially explain the suggested improved therapeutic index resulting from time-modified continous infusion of FdUrd.

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CIRCADIAN TIME-DEPENDENT TISSUE UPTAKE OF CISPLATIN (CDDP) AND CARBOPLATIN (CBDCA) FOLLOWING REPEATED ADMINISTRATIONS: RELATIONSHIP WITH DRUG TOXICITIES IN MICE

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SUMMARY

Dosing time-dependent platinum (Pt) toxicities is partly related to circadian rhythms in urinary, plasma and tissue pharmacokinetics. Lowest tissue Pt concentrations following a single dose of CBDCA were found at 16 hours after light onset (HALO), time which corresponded to least toxicity.
Total Pt accumulation in main target tissues (kidney and colon) was 3-4 fold higher following repeated weekly venous injections of sublet'al doses of CDDP (5mg/kg) or CBDCA (50mg/kg). Tissue Pt uptake was ˜twice less after CDDP or CBDCA dosing at 16 HALO than at 8 HALO (p≤0.01).

KEY WORDS: platinum complexes, circadian rhythm, pharmacokinetics, toxicity.

INTRODUCTION

Murine tolerance for antitumor Pt complexes exhibit a similar circadian rhythm despite different organ-specific toxicities. Least toxicity occurred when a potentially lethal dose of CDDP or new analogs such as CBDCA and oxaliplatin (1-OHP) were administered near the middle of dark-activity-span of rodents (1-3).
Such circadian rhythms in host toxicity were maintained despite eight repeated weekly i.v. administrations of non lethal doses of CDDP or CBDCA. CDDP-induced renal and hematologic toxicities were cumulative, whereas CDBCA-associated bone marrow suppression was reversible (4). Optimal tolerance occurred when either drug was injected near the middle of the activity span of mice (16 HALO). The present investigation aimed at assessing whether tissue drug disposition was involved in the mechanisms of these circadian rhythms of toxicity.

MATERIALS AND METHODS

1. Animals and synchronization

Male B6D2F1 were housed 3 per cage, with free access to food and water, and were synchronized 3 weeks prior to injection in a chronobiologic animal facility with an LD12:12 lighting regimen. Each facility has six compartments, each one having its own programmable lighting regimen. Synchronization of mice was checked prior to injections by measuring rectal temperature.

2. Study design (fig. 1)

3. Tissue Pt dosage

Mice were sacrified q.2 weeks and blood, spleen, kidney and colon were sampled. Tissues were immediately weighed, and then air-dried to constant weight. Dry tissues were digested in 1ml of nitric acid during 1hr at 100°C. After acid evaporation Pt tissue content was measured by flameless atomic absorption spectrophotometry (FAAS) with electrothermal atomization. The instrument used was a Perkin-Elmer Model 2280; the following time temperature program was used: drying for 30s at 120°C, ashing for 30s at 1400°C, atomization for 3s at 2700°C, and purging for 2s at 2700°C.

RESULTS

Platinum distribution in tissues

Pt concentrations were detectable in all tissues studied except in plasma.

Spleen

Mean splenic Pt concentration (μg/g dry tissue) increased ten fold between day 14 (4.6) and day 42 (47.5) with CDDP and 2.5 fold from day 14 (8.3) to day 56 (20.7) with CBDCA. Highest splenic Pt concentrations resulted from CDDP dosing at 8 HALO (p from ANOVA <0.001). No such effect was validated for CBDCA.

Kidney

Pt concentrations in kidney increased five fold between day 14 (3.0μg/g) and day 42 (15.9μg/g) with CDDP. However, CBDCA-associated Pt accumulation over this time frame was modest although statistically significant (from 2.6 to 4.1μg/g). Pt tissue uptake was twice less following CDDP or CBDCA dosing at 16 HALO as compared to 8 HALO (p from ANOVA <0.01) (fig. 2).

Colon

The evolution of Pt uptake in colon was similar to that observed in kidney. Mean Pt concentration increased five fold between day 14 (1.6μg/g) and day 42 (7.9μg/g) with CDDP. A near doubling of colon Pt concentration was observed with CBDCA over this time frame (from 1.4 to 3.1μg/g). Pt concentration in colon was twice less following the injection of either drug at 16 HALO as compared to 8 HALO (p from ANOVA <0.01).

DISCUSSION

Pt accumulation in all three tissues was much higher following repeated

SCHEME OF STUDIES

288 male B6D2F1 mice were injected i.v. weekly (↓) with CDDP (5 mg/kg/wk)
or CBDCA (50 mg/kg/wk) at either of 3 circadian dosing times (0,8 or 16
hours after light onset). Blood and tissues were sampled every 2 wks (↕) in
72 mice (12 per drug and per time-point).

Fig. 1   Injection Time (days)
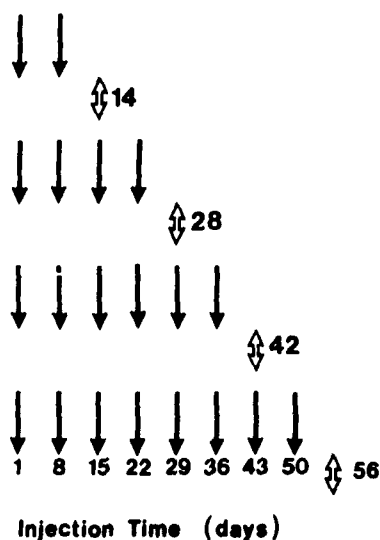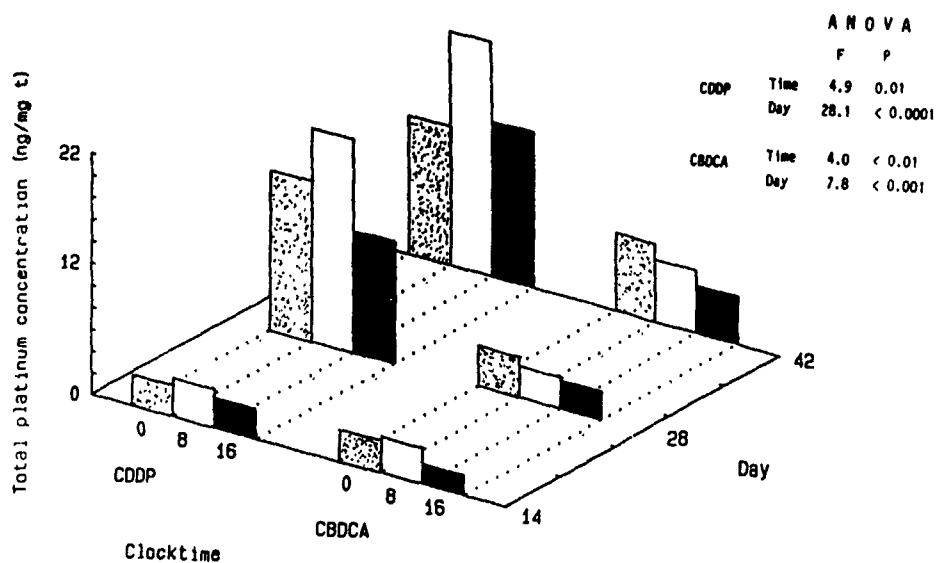
FIG. 2 Histogram of mean platinum concentration in murine kidney.
Mice were injected i.v. weekly with CDDP (5 mg/kg/wk) or CBDCA
(50 mg/kg/wk) for 6 wks at either of 3 circadian dosing times (0,8
or 16 hours after light onset). Kidney was sampled every two weeks
(day 14, 28 and 42) in 60 mice (10/drug/time-point).

dosages of CDDP as compared to CBDCA. This paralleled the
toxicokinetics with regard to effects of both repeated dosages and
dosing time of either analog. Tissue elimination of Pt ion was faster
after CBDCA injection than after CDDP both in rodents (7,8) and in
rabbits (9). Furthermore, both Pt uptake and tissue toxicities were
least following CDDP or CBDCA dosing at 16 HALO despite their different
pharmacokinetic properties. The latter may relate to the increased
chemical stability of CBDCA and its relative lower binding to plasma
proteins as compared to CDDP (5,6). Dosing either Pt complex at the
"right" time appears as even more critical if administrations are to be
repeated. The ability of target tissues to take up the drug contribute
in a large part to the stable mechanisms of Pt toxicities.

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A CHRONOPHARMACOLOGIC STRATEGY FOR THE DEVELOPMENT OF THE NEW ANTICANCER AGENT OXALATOPLATINUM (1-OHP): TOWARDS ITS CONDITIONING INTO CHEAP, DISPOSABLE AND PROGRAMMABLE-IN-TIME MICROPUMPS

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ABSTRACT

1-OHP is a third generation platinum complex which exhibits similar or increased antitumor effectivenes as compared to cisplatin. Preclinical studies have shown a circadian rhythm in both drug toxicities (survival, body weight loss, neutropenia, bone marrow and jejunal necrosis) and plasma and tissue pharmacokinetics. Drug uptake was highest in spleen and kidney and lowest in the gastrointestinal tract. Highest platinum uptake corresponded to 1-OHP dosing at 8 hours after light onset (HALO), e.g., the most toxic time. A randomized phase I trial comparing infusions at constant (sch.A) vs circadian-modulated (sch.B) rate (with peak at 16.00 hr) was undertaken in 24 pts with breast or liver ca. Pts' numbers and characteristics were similar in both groups. Sch.A was associated with a higher incidence of neutropenia (19% vs 2%; $p \leq 0.01$), paresthesias (28% vs 2%; $p \leq 0.01$) and emesis (42% vs 27%; $p = 0.15$) as compared to sch.B. Mean dose given was higher in sch.B (155 mg/m^2) than in sch.A (130 mg/m^2) (p ≤ 0.001). It was attempted to isolate a special activity profile for this drug by taking advantage of the ability of platinum complexes to modulate 5-fluorouracil (5-FUra) cytotoxicity. 41 pts with metastatic colorectal cancer were treated in a phase II clinical trial aiming at assessing the activity of a three-drug combination chronotherapy protocol (5-FUra: 3500 mg/m^2, folinic acid· 1500 mg/m^2, 1-OHP: 125-150 mg/m^2 per course). A complex drug delivery schedule was devised, based on preclinical studies. A programmable-in-time multichannel ambulatory pump (Intelliject, Aguettant, France) was used to administer such complex delivery schedule over 5 days every 3 wks to outpatients; 26/41 pts (63%) exhibited a 50% reduction in tumor size irrespective of prior therapy These results are > twice those achieved by other regimens. 1-OHP will soon

be delivered in recently developed disposable programmable
in time micropumps.

KEY WORDS

Drug development ; circadian chronotherapy; platinum complex;
cancer.

GLUTATHIONE IN HUMAN BONE MARROW AND ITS CIRCADIAN STAGE RELATION TO DNA SYNTHESIS

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ABSTRACT

Glutathione (GSH) content and its circadian stage dependent relation to fraction of cells in DNA synthesis in human bone marrow samples was investigated. From each individual bone marrow samples were collected every 4 hours during a 24-hour period, altogether 7 samples. Cell cycle distribution was analyzed by flow cytometry. In addition, reduced (GSH) and oxidized (GSSG) glutathione were determined. The mean GSH content during the sample periods varied from 1.94 to 3.27 nmol/mg protein between the subjects. There was a marked circadian stage dependent variation of GSH content in all individuals. The mean percentage of cells in the DNA synthesis-phase varied from 10.6% to 14.5% between the individuals, but with an intraindividual circadian stage dependent variation from 73.6% to 274.0%, when comparing the highest value to the lowest. When correlating each time point for both parameters for the pooled data according to circadian stage, a close circadian stage dependent covariation between GSH content and DNA synthesis-phase was demonstrated, with the lowest value of GSH occurring a few hours before midnight and DNA synthesis at about midnight. The myelosuppressive effect of many chemotherapeutic agents assumed to be detoxified by GSH dependent mechanism(s) should be considered in the light of the low GSH content in the human bone marrow and its circadian stage relation to DNA synthesis. The larger circadian stage dependent variation seen in the DNA synthesis, indicates that the circadian varying proliferative status of the bone marrow may be the most important single factor of these two to be taken into account for drugs mostly affecting the DNA synthesis.

KEYWORDS

Glutathione, DNA synthesis, human bone marrow, circadian.

INTRODUCTION

Glutathione (GSH), a cystein-containing tripeptide, has been assigned an important role in the cellular defence against free radicals and oxidative injury, detoxification processes and in the protection of the cell against radiation damage (Lee et al., 1987). It is the most abundant intracellular non-protein thiol and the average cellular content amounts to 0.5-10 mmol/L (Meister and Anderson, 1983). It has been demonstrated that depletion of intracellular GSH in vitro enhances the cytotoxicity of several chemotherapeutic agents. In addition, glutathione has a radioprotective effect.
It has also been shown that tumor cells obtained from a patient with ovarian adenocarcinoma after the onset of resistance, had significantly higher level of GSH as compared to the level before onset

of resistance. In vitro studies have further demonstrated that resistant cells can be sensitized by depleting GSH using the metabolic inhibitor buthionine sulfoximine (BSO) (Lee et al., 1987). This shows that pharmacological modulation of cellular GSH content may alter the sensitivity towards several cytotoxic drugs, pointing to the possibility that physiological changes in cellular GSH content may be an important determinant for the susceptibility of both normal and malignant cells to such agents.

Cell cycle phase-dependent differences in the effects of cytotoxic drugs are well documented. Rapidly proliferating cells are usually more sensitive to cytotoxic drugs than quiescent cells.

In current cancer treatment bone marrow suppression represents the main dose limiting factor. It was therefore of interest to measure the level of and a possible circadian variation of glutathione content in the human bone marrow in order to evaluate its detoxifying capacity, and relate it to the circadian stage dependent proliferative activity, i.e. the DNA synthesis-phase.

MATERIALS AND METHODS

From February 1988 to August 1988 we investigated GSH content and fraction of cells in DNA synthesis in the bone marrow of 10 healthy male volunteers (mean age = 30.7 years; range 19 - 43 years). The volunteers had given their informed consent to enter the study, which was performed according to the guidelines of the regional medical ethics committee.

All the individuals followed a regular diurnal rhythm for at least 3 weeks before the experiment. They continued their usual activities during the study period, apart from the sampling periods. Their regular diurnal rhythm was validated by the determination of cortisol levels every fourth hour, which showed the usual circadian pattern for all individuals. Bone marrow was sampled every fourth hour during one 24-hour period, altogether 7 times. Bone marrow was drawn by puncturing the sternum and anterior iliac crests. In order to exclude that variations should be attributed to dilution of the samples, caused by local bleeding at the puncture site, differential counts were performed on smears from all individual samples. No samples had to be discarded because of peripheral blood admixture (results not shown). Bone marrow (0.2-0.4 ml) was aspirated into a 2-cc syringe and two droplets of the aspirated sample were immediately put into liquid nitrogen for determination of GSH.

Another part of the bone marrow was stained for flow cytometry by adding it to 2 ml of ice-cold staining solution consisting of ethidium bromide, detergent and RNAse, according to the method described by Vindelöv (Vindeløv, 1977). The tube was sealed and the solution shaken before being placed in an ice-bath for at least 10 minutes. The bone marrow cell suspension was analyzed on a Cytofluorograf 50H (Ortho), interfaced to a Model 2150 Computer. Cell cycle distribution was estimated using the cell cycle analysis program . The mean CV (coefficient of variation) of the DNA histograms was 3.3 %.

For GSH determination bone marrow (liquid nitrogen) was extracted within 3 days after sampling with 1 ml of ice-cold 5% sulfosalicylic acid and the precipitated protein removed by centrifugation. This protein precipitate was taken care of for subsequent protein determination.

Briefly, the GSH was determined in the acid extract by derivatizing the free sulfhydryl groups with monobromobimane, and the GSH-bimane derivative was quantitated by chromatography on a 3-μm ODS Hypersil column, which was equilibrated and eluted with 14.2% methanol in 43.5 mM Na-acetate, pH 3.9 . The column was washed by increasing the methanol concentration to 90%. The retention time of the GSH-bimane derivative was 3.2 min.

RESULTS

The average GSH content from seven consecutive bone marrow aspirations during a 24-hour period varied from 1.94 to 3.27 nmol/mg protein between the different individuals, i.e. a difference of 68.6% between the lowest and highest individual average GSH content. The average value for the fraction of cells in DNA synthesis-phase at the 7 time points during the 24-hour sampling period measured by flow cytometry varied between the subjects from 10.6% to 14.5%.

For the individual subjects we observed a circadian stage dependent variation in reduced glutathione from 31.0% to 90.2%, as compared to the lowest value. When the data from all subjects were pooled there was a trend towards increasing GSH content from midnight till 12.00 pm., with a marginally significant difference between the GSH content at 24.00 hours compared to 12.00 hours. By cosinor analysis this circadian variation was also marginally statistically significant (p=0.08) with acrophase found to be in late morning, and trough a few hours before midnight.

The individual subjects also demonstrated a circadian dependent variation of the DNA synthesis-phase, with a mean variation from 73.6% - 274.0% relative to the lowest value. For the DNA synthesis an increase from midnight until 16.00 hours was observed for the pooled data, with a statistically significant lower value for the DNA synthesis at 24.00 hours as compared to 16.00 , 12.00 and 08.00 hours. For 9 of 10 subjects there was a covariation between GSH concentration and DNA synthesis-phase according to circadian stage, either demonstrating a near-identical covariation or a slightly phase-shifted near-identical covariation. This is shown in Fig.1 for four individuals; both parameters demonstrating the same phasing at most of the time points, or a different phasing in time of ± 4-8 hours.
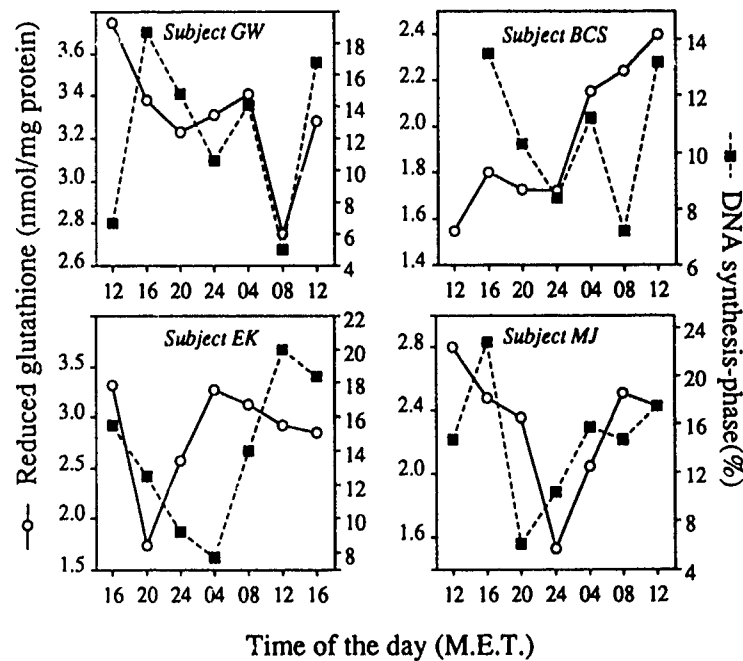
Fig.1. Circadian stage dependent relation between DNA synthesis and reduced glutathione content in four individuals.

When adjusting for the phase difference for five of the ten subjects, we found a close correlation between each subject's GSH content and DNA synthesis-phase, with a statistically significant correlation for the pooled data (r = 0.36, p = 0.004, n = 63). For seven of ten subjects an even closer relationship was demonstrated when one highly deviating time point during the 24-hour period was not taken into consideration. This was done because in these cases *all but one* time point demonstrated a near-identical circadian stage dependent covariation or a near-identical slightly phase-shifted circadian stage dependent covariation between GSH content and DNA synthesis-phase. The pooled GSH values showed a highly significant correlation with the DNA synthesis-phase when discarding the few abovementioned time points (r = 0.63, p < 0.0001, n = 54).
The curves for the pooled data according to circadian stage demonstrated a close covariation for the average GSH content and DNA synthesis-phase. A peak value for the GSH content was observed at time 12.00 hours 24 hours apart, and was followed by a decrease in GSH content during the afternoon. This may indicate a slightly different phasing for the GSH content relative to the DNA synthesis, because this parameter reached its highest value 4 hours later, i.e. at time 16.00 hours. The small difference in phasing is supported by the data of some of the individuals.

DISCUSSION

Glutathione and glutathione dependent enzymes are established as important factors in the defense of several normal and malignant cell types against cytotoxic agents. Morover, the sensitivity of both normal and malignant cells to cytotoxic agents is partly related to a high proliferation rate (Smaaland et al., 1989). We measured reduced and oxidized glutathione in the bone marrow cells. Less than 10% of the glutathione content in bone marrow was oxidized. This implies that the circadian variations do not reflect different degrees of oxidation of glutathione in vivo. It also excludes improper sample handling causing glutathione oxidation as a source of erratic results. The circadian changes in total glutathione content may thus be due to variations in glutathione biosynthesis and utilization.

We found a low mean GSH content in human bone marrow from repeated measurements over a 24-hour period, which is in accordance with murine data reported earlier. Five to more than ten times higher levels of GSH have been measured in other normal organs in rodents compared to the bone marrow, the difference being even greater compared to malignant tumors. This may suggest limited capacity of the GSH-dependent detoxification mechanisms in bone marrow, which should be related to the high sensitivity of human bone marrow to many cytotoxic drugs.

Our data demonstrate a close or slightly phase-shifted circadian stage dependent covariation of GSH content and fraction of cells in DNA synthesis-phase in the human bone marrow within the same subjects. In addition, evaluation of the pooled data showed a covariation between the *mean* values for GSH content and DNA synthesis according to circadian stage. These findings are thus in agreement with earlier findings demonstrating a relation between these two parameters when cells are activated into cell cycle progression or are in exponential growth (Shaw and Chou, 1986).

The measured circadian stage dependent variations in GSH content in the individual human bone marrow are rather large, and seem to be related to cell proliferation. It could thus be considered a possible determinant of bone marrow suppression induced by cancer chemotherapy in humans. However, the near-identical circadian stage dependent covariation between GSH content and cells in the DNA synthesis-phase might theoretically neutralize each other. Which one of these parameters will be the most important, may depend on the actualiy cytotoxic drug used. The larger circadian stage dependent variation seen in the DNA synthesis, indicates that the proliferative status of the bone marrow may be the most important single factor of these two to be taken into account for drugs mostly affecting the DNA synthesis.

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CHRONOTOXICITY AND PHENOTYPIC VARIABILITY

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ABSTRACT

The Chronotoxicity of anticancer drugs was studied in C57BL/6J mice.
Cisplatinum (Cis), Adriamycin (Adria), and Cyclophosphamide (Cyclo)
were injected, separately, at four circadian stages to groups of
mice. Toxicity was monitored by body-weight and mortality. The
results were compared to those of a parallel study with BALB/c
strain (recently reported). Cyclo induced minute body-weight
changes in both strains. Adria did not produce significant body-
weight loss in BALB/c while a significant time dependency of body-
weight loss was affected in C57BL/6J. Both strains did exhibit
significant, but different, time dependent patterns of body-weight
loss and recovery following Cis administration. In C57BL/6J
chronomortality was affected by Cis and Cyclo and time independent
mortality was caused by Adria. None of the drugs had caused any
lethal effects in BALB/c.

KEY WORDS

Chronotoxicity; anticancer drugs; genetic heterogeneity.

INTRODUCTION.

Only few studies were carried out with regard to the genetic
variability of rhythms in mice strains. Among them are light
preference (Matsuo and Tsuji, 1989), free running of locomotor
activity (Abe et al., 1989; Beau, 1988), melatonine content (Goto et
al., 1989), enzymes' activities (Peleg et al., 1982; Feuers et al.,
1984) and drug sensitivity (Levi et al., 1988). As most strains of
the same species are exposed to the same external stimuli, their
phenotypic temporal order will be similar even if genetic
differences exist in their endogenous rhythms. Drugs did not
participate in the natural selection process of mammals and their
effects were not programmed in the adaptive expression of the
temporal order. Thus, genetic variability may yield different
chronotolerance phenotypes to the same drug in different strains and
clinical chronotherapeutic schedules which are based on experimental
models involving only one rodent strain, may be misleading.
In the present study we examined the chronotoxic effects of three
commonly used drugs, Cisplatinum (Cis), Adriamycin (Adria) and

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cyclophosphamide (Cyclo) administered at different times to C57BL/6J
mice and the results were compared to the results of a parallel
study which was carried out with BALB/c strain (Peleg et al., 1989).

MATERIALS AND METHODS

C57BL/6J mice, 3 - 5 months old, were housed 5 - 10 per cage
(36x32x17 cm) with food and water freely available. Lighting
regimen, prior and during the experiments, consisted of alternating
cycles of 14 hcurs light (onset at 0600) and 10 hours dark (L:D
14:10). Light intensity at cage level was 350 Lx. Three drugs were
used: Cisplatinum (Abiplatine, Abic, Israel), 1.6 mg/kg. Adriamycin
(Adriablastina, Farmitalia, Italy) 1 mg/kg. Cyclophosphamide
(Cytophosphan, Taro, Israel) 16 mg/kg. Doses were adjusted to mouse
body weight based on human therapeutic doses. Each drug was
administered daily, I.P., to 40-60 mice for 10 to 15 consecutive
days. Each group (10-15 mice) was injected at either 0900, 1500,
2100 or 0300. An additional group of 10-15 mice served as control.
Experiments were repeated 3 to 5 times. Toxicity was monitored by
body weight change (% of original weight) and mortality(%) was
assessed for at least two weeks after the last death in the group.
Results' significance were determined by Chi-square and t-test.

RESULTS

Body-weight loss
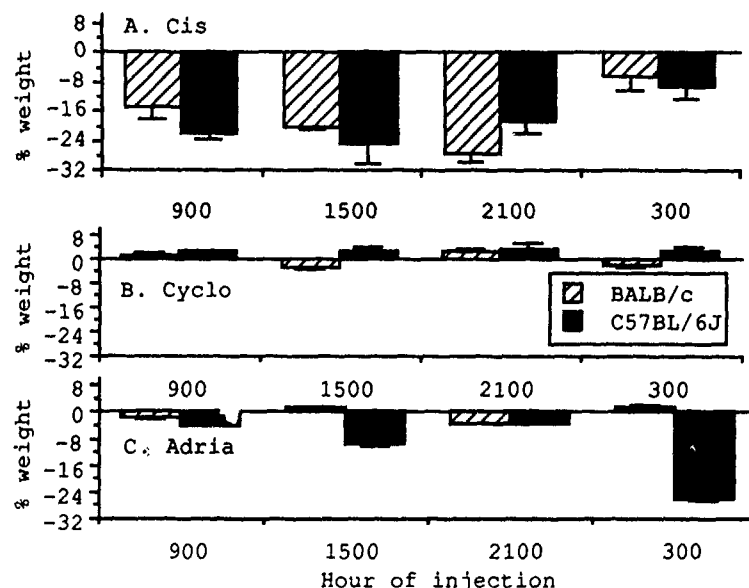
Fig. 1. Percentage of original weight (mean ± s.d) as recorded on
the third week after the first injection of drugs.

Fig. 1 exhibits the chronotoxicity of the drugs as gauged by body-
weight change three weeks after the first injection. Administration
of Cis induced a time dependent body weight loss in C57BL/6J. The
smallest effect was observed when the drug was injected at 0300
(loss of 9.5% of initial weight, Fig. 1A) significantly (p<0.01)

differing from the loss at all other injection times. Maximal loss (24%) was recorded in the group injected at 1500 differing slightly, yet significantly (p<0.05), from loss recorded in those injected at C900 and 2100.(Fig. 1A). BALB/c mice, injected with Cis portrayed a similar time dependency and magnitude of body-weight loss to those observed in C57BL/6J. However the patterns' phases differed (p<0.05) between the two strains (Fig. 1A). Three weeks after injections onset, Cyclo (Fig. 1B) induced very small weight changes in both strains, while Adria induced moderate loss (10%) in C57Bl/6J mice injected at 1500 and severe loss (26%) w.en injected at 0300 (Fig.1C). These two groups differed from each other (p<0.01) and from the C57Bl/6J mice injected at 0900 and 2100 (p<0.05-0.001) .At that time, hardly any weight loss was detected in all BALB/c groups injected with Adria and its time-dependent effects were evident only on the seventh week. The least tolerant hours (moderate effect at 2100 and severe at 0900) differed from the least tolerant hours recorded in C57BL/6J mice(Peleg et al., 1989).Fig. 2 demonstrates the body-weights of the two strains during nine weeks after injection of Cis at 1500. The two strains differ from each other in the kinetics of weight loss and in their recovery patterns.
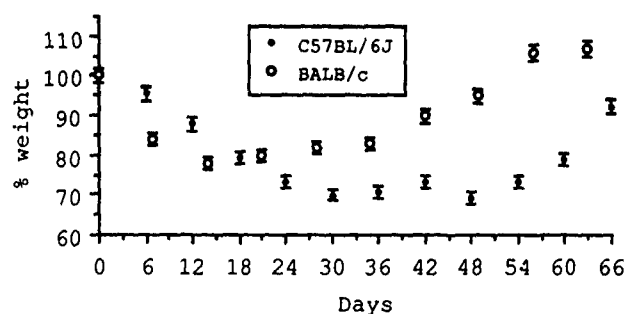
Fig. 2. Pattern of Body-weight change (mean ± s.e) following injections of Cis at 1500.

Mortality

Table 1. Chronomortality (%) of C57BL/6J following injections of drugs.

Injection times	% Mortality			
Drugs	0900	1500	2100	0300
Cis	35	50	20	50
Adria	50	50	50	50
Cyclo	0	50	50	41

Chronomortality patterns (Table 1) were observed in C57BL/6J mice upon the administration of Cyclo and Cis (p<0.05-0.01) while time independent mortality was induced by Adria injections (Table 1). None of the drugs induced mortality in the BALB/c strain at any injection time (Peleg et al., 1989).

DISCUSSION

The results clearly demonstrate the existence of a strain-related
tolerance and chronotolerance to each of the examined drugs. Strain
specific tolerance was revealed by the fact that therapeutic doses
of these drugs induced mortality only in BALB/c (table 1).
Furthermore, The strains exhibited different time dependent patterns
of body-weight change following drugs injections. The phases of the
chronotoxic and the chronorecovery patterns induced by Cis
portrayed strain specificity (Fig. 1A and Fig. 2). The difference in
the response of the strains to Adria was multifaceted. In C57BL/6J
the chronotoxic pattern expressed itself immediately while in BALB/c
the chronotoxic pattern emerged only after seven weeks. In addition
the two patterns differed with regard to their phases (Fig.1C).
Extrapolation of results from murine studies to clinical treatment
is usually practiced by phasing the chronotoxic pattern to the
rhythms of variables like locomotor activity and temperature. The
rhythms of such variables have been programmed throughout the
evolutionary processes and, due to their important adaptive value,
different strains may express identical rhythms of these variables.
On the other hand, the selection of drug tolerance rhythms was not a
part of the evolutionary process. As documented in the present
study, chronotoxic patterns induced in two mouse strains, by the
same drug, differed from each other. Thus, in order to optimize
chronotherapeutic procedures, these observations forward the need
to: a) design a decision policy with regard to the choice of time-
dependent drug application which sho ld be based upon screening many
strains and additional reference rhyt ms, and b) to examine the
variability of chronotoxicity (for each drug) in humans.

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ABNORMAL PATTERNS OF PLASMA CORTISOL IN BREAST CANCER PATIENTS

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ABSTRACT

Circadian variations of cortisol were studied in 13 patients with advanced breast cancer. The secretory profiles were found abnormal in 8 out of the patients with either higher mean values or flattened profiles and/or shift in the peak or trough location.

KEY WORDS

Cortisol ; breast cancer ; circadian rhythm ; desynchronization

INTRODUCTION

A number of studies have reported an increase of plasma cortisol concentrations in some patients with breast cancer. The question then arises whether this increase may be related to an alteration of the liver metabolism of the hormone, especially in patients with advanced cancer and liver metastasis and whether it is accompanied with a perturbation of the circadian rhythm of cortisol.
Since previous studies have shown a modification of the circadian rhythmicity of CEA in cancer patients (Touitou et al. 1988), we found worth documenting the circadian rhythmicity of plasma cortisol in women with advances breast cancer. 13 women (36-73 yrs) with histologically proven metastatic breast adenocarcinoma and measurable disease volunteered for the study. Metastatic sites included liver (7/13), bones (8/13), lung (3/13). The main characteristics of the patients are displayed on Table1. Blood was sampled every 4 hours over 36 or 48 hours on each patient.

RESULTS

Whereas six patients showed the usual rhythmicity of plasma cortisol with a peak located around 08h00 and a trough around 00h00, the seven others had an alteration of the rhythm with either a shift in the peak timing and/or high concentrations between 04h00 and 12h00 and or a flattened profile. The cosinor analysis allowed to show a group rhythmicity with the following characteristics :
P < 0.002 ; Mesor : 10.2 $\pm$ 0.54 mg/dl ; Amplitude : 2.74 $\pm$ 1.85 mg/dl ;
Acrophase : 9.38 $\pm$ 2.53.

Table 2. Abnormalities in the peak and trough location of plasma cortisol

	Peak		Trough	
Patient	µg/dl	time	µg/dl	time
JL	16.2	08h	5.2	04h
JE	20.6	08h	14.8	04h
MTM	33.2	08h	3.6	04h
NC	13.7	08h	0.8	20h
DS	14.6	16h	0.3	04h
DB	32.9	00h	8.1	04h
BR	16.9	00h	2.8	04h
MP	15.6	04h	1.6	00h
DM	20.9	12h	4.0	00h
SF	17.6	08h	1.6	00h
FC	15.0	08h	6.2	00h
GS	6.4	08h	0.9	00h
LC	22.9	08h	8.7	00h

DISCUSSION

Cortisol is a hormone considered as a strong oscillator and therefore as a marker of the circadian rhythmicity in man (Touitou et al). We report here changes in the pattern of plasma cortisol in breast cancer patients suggesting a rhythm desynchronization.
The circadian profiles of cortisol in breast cancer patients were found normal in 5 patients although one of these patients has a so called normal pattern but low levels

Table 1. Characteristics of patients and basal values of serum tumor marker antigens, total proteins and cortisol. Each value represents the mean of samples at 08h during 2 consecutive days.

Patients	Age (years)	Metastatic localization	Prior DOX/THP* (mg/m2)	CA 15-3 U/ml	CEA ng/ml	Proteins g/l	Cortisol μg/dl
1 JL	50	liver, skin, nodes	350/0	86	9.1	51	17.8
2 SF	57	bones, skin pleura + breast	270/0	490	336	66	14.2
3 JE	73	liver bones, bone marrow	500/0	54	4.3	73	18.5
4 DS	47	liver bones, lungs, nodes, pleura	400/180	275	28.4	55	3.0
5 MP	44	liver, bones	450/0	96	123	66	14.0
6 DB	60	liver	0/800	68	2.4	79	25.5
7 MTM	58	liver,bones	400/0	212	18.5	54	22.5
8 FC	52	skin, nodes, lungs choroïd	100/0	56	7.6	68	10.3
9 BR	50	liver	0/60	440	109	67	12.9
10 GS	45	bones	395/0	242	52	64	6.1
11 NC	48	bones	160/0	430	49.5	72	14.0
12 LC	57	bones	200/0	212	7.6	74	23.5
13 DM	36	local recurrence	330/0	46	18.2	79	8.4
Limits of usual values				< 37	< 5	65-80	12-25

* DOX = Doxorubicin, THP = 4'-O-tetrahydropyranyl Doxorubicin

of the hormone (patient GS, Fig. 1). Eight out of the 13 patients showed an abnormal pattern of plasma cortisol (Fig 2).

These abnormalities were various e.g. flattened profile, shift in the peak time, shift in the trough time, plateau of high value in the morning. These abnormalities are coherent with the hypothesis of a rhythm desynchronization in these patients as already abserved in other types of cancers (Touitou et al. 1988, Focan et al. 1986).
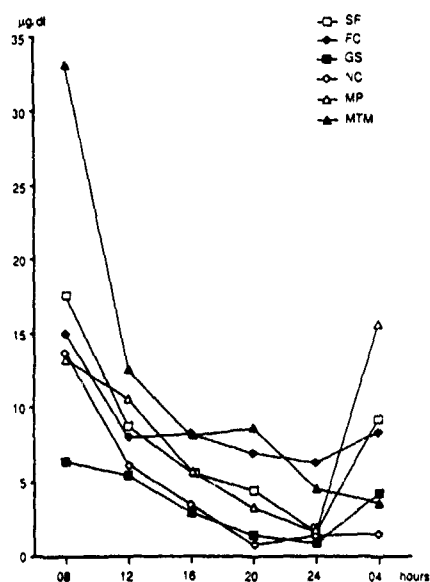
Fig 1 Normal patterns of plasma cortisol in breast cancer
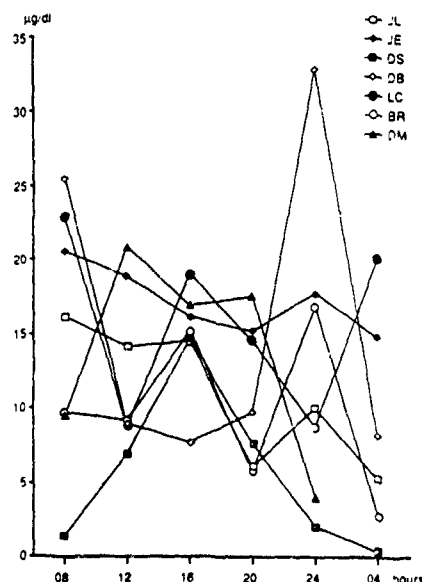
Fig 2 Abnormal patterns of plasma cortisol in breast cancer

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SECTION 6

GENERAL
CHRONOPHARMACOLOGY

Chairpersons: D. Kripke and B. Bruguerolle

CIRCADIAN RHYTHM OF PAIN IN MAN: STUDY BY MEASURE OF NOCICEPTIVE FLEXION REFLEX

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ABSTRACT

The aim of this work is to study the circadian changes in physiological pain in man by the objective measure of noceptive flexion reflex. The nociceptive reflex threshold is established in five men every four hours in the day. The measure is realized with the Algometer E3A prototype, which is able to spontaneously integrate and analyse electrophysiological responses. During the same experiment, each volunteer evaluates at each six times his pain sensation according to a numerical peak from zero to ten. The chronogram shows a circadian rhythm of nociceptive reflex threshold. The acrophase occurs in the middle of afternoon. The chronogram established from the numerical verbal scale of subjective pain sensation shows a circadian variation in opposite phase with the previous chronogram. The more nociceptive reflex threshold is reduced, the more the pain sensation is important.

KEY-WORDS

Pain, circadian rhythm, noceptive flexion reflex, electrophysiology, man.

INTRODUCTION

Circadian rhythm of pain has been already described by many authors (Jores and Frees, 1937 - Procacci et al., 1974 - Reinberg and Reinberg, 1977). But, these estimations have been made with subjective measures or pharmacological methods. Recently, it has been shown that the measure of nociceptive flexion reflex is an objective method for pain evaluation (Willer, 1977, 1985).
This author has established, in many works, the existence of fair correlation between this method and classical measures of quantitative psychology.
So, it appears interesting to study the circadian change of psychological pain in man with this objective method.

MATERIALS AND METHODS

In five men of an average of 30 years old, without physical defect and living in elementary conditions of social synchronization (08.00 - 23.00), standardized dietetic meals at 13.00 and 20.00, an evaluation of nociceptive reflex threshold is established every four hours (13.00, 17.00, 21.00, 01.00, 05.00 and 09.00) during twenty four consecutive hours.

This measure is realized with the Algometer E3A prototype, which is able to make spontaneously the integration and analysis of electrophysiological responses. The electric nociceptive stimulation is made at sural nerve level; the nociceptive reflex response is registred at the femoral biceps muscle.

Self-adhesive electrode affixing on these stimulations and registring spots allow repeated evaluations without night mobilization. Each measure has included a minimum of 30 stimulations with variable intensity. Each activity reflex is controlled by an oscilloscope. A regression straigth line is established from these data. The nociceptive reflex threshold is determinated as the abscisse value corresponding to the intersection between the regression straight line and the perpendicular line corresponding to the ten percent value of the Y-axis to discard basal electromyographic activity.

During the same experiment, each subject has evaluated at each similar time his pain sensation according to a numerical scale from zero to ten.

RESULTS

The reference value is always 100 at 13.00 for each patient; all other values are expressed as percentage of this reference value. For each subject, a circadian change in pain is established; the reflex threshold is decreasing during the night.

For these five men, the chronogram shows a large decrease in reflex threshold at 01.00 (-20% of initial value) (figure 1). The acrophase occurs in the middle of the afternoon (17.00).

The chronogram established from the numerical verbal scale of subjective pain sensation shows a circadian variation in opposite phase with the previous chronogram (figure 2).

The more nociceptive reflex threshold is reduced, the more pain sensation is important. So, the acrophase of pain perception is at 05.00 (6.00 $\pm$ 0,95) and the bathyphase between 09.00 and 17.00 (3,6 $\pm$ 0,25).

DISCUSSION

This result confirms a circadian rhythm in physiological pain. The explanation of this physiological variation is considered in many neurophysiological studies. Frederickson et al. (1977) shows, in mice, that the morphinic analgesia effectiveness follows a circadian rhythm, synchronous of the circadian rhythm of physiological pain so thus, the circadian variation of hyperalgia induced by naloxone injection.

Moreover, in mice, the beta endophin rate in pituitary and some brain nuclei were increased when the threshold sensation of pain is important in active period (Kerdelhue et al., 1983).

So, the endogenous nature of circadian rhythm of pain can be confirmed. Because of the excellent correlation of such an objective measure of pain with the subjective evaluation of pain, this study shows the interest and the validity of this nociceptive flexion reflex method to quantitatively and objectively evaluate pain in man.
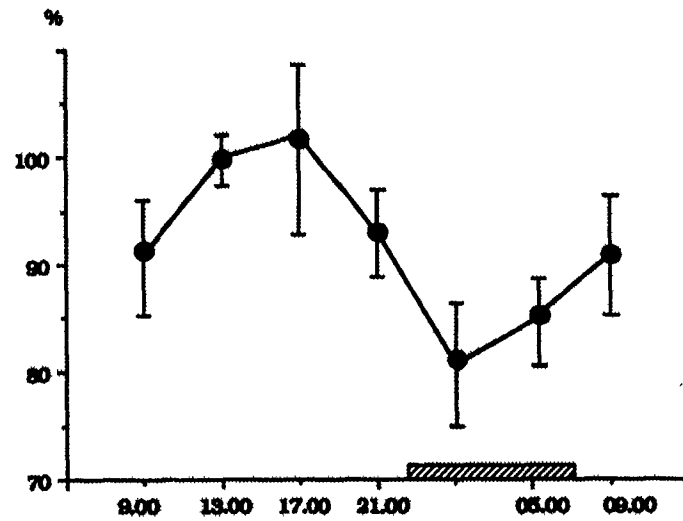
Fig. 1. Circadian changes of nociceptive flexion reflex expressed as percentage of the reference value (100% at 13 h).
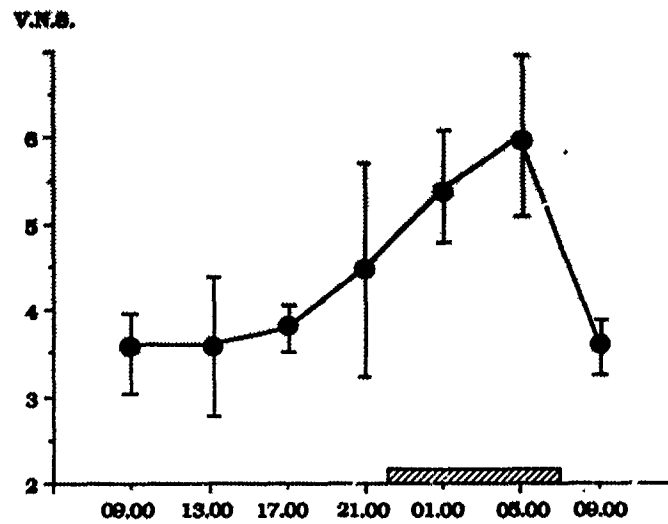
Fig. 2. Evaluation of circadian changes of pain sensation with a verbal numeric scale from 0 to 10.

CONCLUSION

This work confirms the existence of circadian rhythm in physiological pain, perception with a large decrease in threshold pain during rest period. It shows the interest of the nociceptive flexion reflex method to evaluate quantitatively and objectively pain in man and it appears as a very important new technic for the clinical and pharmacological evaluations of pain in man.

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TEMPORAL VARIATION IN THE SELF-ADMINISTRATION OF MORPHINE SULFATE VIA PATIENT-CONTROLLED ANALGESIA IN POSTOPERATIVE GYNECOLOGIC CANCER PATIENTS

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ABSTRACT

The self-administration of morphine sulfate in a sample of 45 post-surgical gynecologic cancer patients using patient-controlled analgesia therapy was studied for 48 consecutive hours starting at 0800 the morning after surgery. Patients did not receive a concomitant continuous basal dose of analgesic in combination with PCA therapy. Single end-to-end cosinor analysis of these data indicated significant 12 and 24 hour patterns in average self-administration of analgesic with peak delivery occurring between 0800-1200 hours.

KEYWORDS

Cancer patient, circadian rhythm, pain, morphine sulfate, patient-controlled analgesia

INTRODUCTION

Serious deficiencies exist in establishing adequate analgesic dosage regimens for the satisfactory relief of postoperative pain (Austin et al., 1980; Graves et al., 1983; Atwell et al.,1984; Speltzer & Anderson, 1987. Factors such as variable absorption rate of injections and delays in analgesic administration by health care personnel have resulted in inadequate analgesia. However, new methods of analgesic administration have become available. Technical advances have led to the development and acceptance of the PCA pump in the treatment of postoperative pain. While it is well recognized that the use of PCA results in a lower total narcotic dose delivered to the patient, little information exists about the optimal utilization of this drug delivery device. Currently, utilization of PCA in the clinical setting involves a static regimen of drug administration

that delivers a predetermined amount of analgesic per unit time period before the patient is "locked out" until the next time interval when another analgesic dose can be delivered. Often this "on demand" regimen is supplemented by a continuous basal administration of analgesic, although minimal evidence exists in the literature to support this practice.

Even with the addition of a continuous basal infusion, the control of postoperative pain may not be complete. Twenty-four hour variation in analgesic pain sensitivity (Graves et al., 1983) and analgesic utilization (Auvil-Novak et al., 1988) has been previously demonstrated. Previous work by the authors suggested the evidence of a circadian rhythm in 19 patients who self-administered narcotic analgesics via PCA pumps while also receiving a constant basal administration of analgesic. The peak demand for analgesic occurred in early morning (0400-0800) with minimal demand noted in the afternoon. Perhaps of even greater importance was the finding that this increased demand during the morning was accompanied by a peak in the number of unmet attempts at self-administration of analgesic due to the pre-programmed "lockout interval" utilized in PCA therapy to prevent over-sedation of patients. Patients were unable to achieve adequate pain relief, despite the use of a continuous basal dose of analgesic.

The results of this previous work suggest that the current methods of pump utilization, which ignore twenty-four hour variation in the patients requirement for analgesia are not optimally effective, even when a basal dose of analgesic is concurrently administered. While this pilot study and others have shown that a circadian variation in narcotic requirement does exist, the means to best translate this to a clinical regimen have yet to be determined. Toward this end and in order to further define the signature in time of postoperative patients, the following study was performed.

METHODS

Data from 45 diurnally active post-surgical cancer patients who utilized PCA therapy alone for the self-administration of morphine sulfate (MS) were reviewed for circadian and other periodicities in the amount of analgesic delivered. Patients ranged in age between 27-81 years; MEAN = 52.26, SD = ±14.19 years. All patients included in the study were admitted on the gynecology service and underwent an exploratory laparotomy through a vertical midline incision.

PCA pump records were collated to calculate the amount of MS delivered per 4-hour interval. For all patients, data collection began at 0800 on the first post-operative day and continued for 48 consecutive hours. Data from continued use of PCA therapy thereafter were not useful due to decreased demand for analgesic during the postoperative recovery period. Those patients with a history of narcotic or substance abuse or those who required high doses of analgesic in the recent past for the alleviation of malignancy associated or any other types of pain were excluded from participation in the study. Patients who received any sedatives other than the prescribed PCA therapy as well as those individuals who worked alternative shift schedules were also excluded from the study. Patients were educated to use of the PCA pump prior to initiation of therapy.

If a loading dose of MS was not administered in the recovery room, than a bolus of 6 mg MS was administered prior to initiation of the PCA pump.

The calculated PCA dose for each individual was equivalent to the body surface area (m^2) or the mg Morphine/Dose using a 1 mg/ml concentration of MS. A ten minute "lockout interval" was programmed between doses, with a maximum of 6 doses per hour permitted. No basal rate or PRN bolus was permitted for these patients. Alterations in dosage for inadequate analgesia or over-sedation was provided in increments of 0.2 cc/dose.

RESULTS

Temporal variability in the average dose delivered was substantiated by (peak-trough) repeated measures t-test ($p < 0.0001$) with peak MS delivery found between 0800-1200. Single end-to-end cosinor analysis of the data confirmed both 12 and 24 hour patterns of rhythmicity in the amount of drug delivered. For $\tau = 24$ hr.: mesor = 16.66 mg., amplitude = 4.06 mg., acrophase = 11:56, $p < 0.0001$. For $\tau = 12$ hr.: mesor = 16.66, amplitude = 4.67 mg., acrophase = 9:33, $p < 0.0001$. The number of unmet attempts at self-administration of MS due to the pre-programmed "lockout" interval did not significantly vary by clock hour when subjected to analysis by (peak-trough) repeated measures t-test ($0.20 > p > 0.01$).
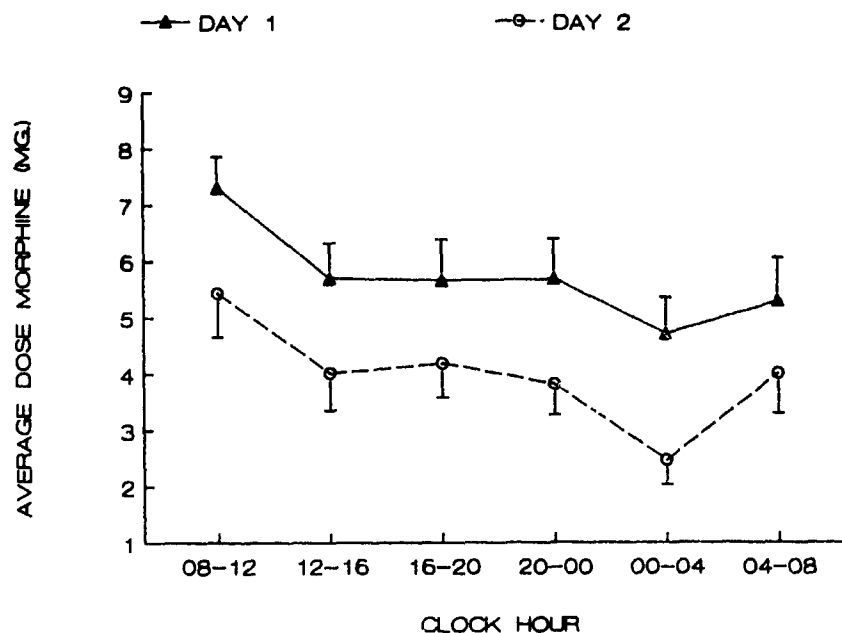
Fig. 1. Average dose of morphine sulfate self-administered by clock hour for the first and second post-surgical day.

DISCUSSION

Previously, the authors have demonstrated temporal variability of self-administered analgesics in post-surgical gynecologic cancer patients receiving MS (Auvil-Novak, et al. 1988). In this prior analysis, patients received a constant basal administration of MS accompanying their self-administered doses of MS via the PCA pump. In contrast to the current study, two differences were found: 1. The previous study demonstrated a significant circadian rhythmicity in the dose of MS self-administered by patients with peak dose delivered between 0400-0800; 2. It was also noted that there was an increased morning demand for analgesic that was unmet due to the "lockout" interval programmed into the PCA pump.

The authors suggest that the results of the current study may identify an effect of basal administration of analgesic that masks the peak analgesic requirement in post-surgical patients. The casual nature of this effect is unknown. The paucity of research papers investigating optimal delivery methods for analgesic therapy suggest that further study of the efficacy of PCA pump utilization and their relation to time-dependent requirements for analgesics would be valuable in adequately meeting patient needs for analgesia.

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CIRCADIAN PHASE DEPENDENCY OF CARDIAC TISSUE LEVELS OF THREE AMIDE-TYPE LOCAL ANAESTHETICS

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ABSTRACT

The aim of this study was to investigate the possible influence of the hour of administration on the heart passage (estimate by the ratio of pharmacokinetic parameters in heart and serum) of three amide-type local anaesthetics : bupivacaine (B), etidocaine (E) and mepivacaine (M). Our data showed significant 24hr variations of the kinetic parameters : highest Cmax ratio was located at 04.00h for B and M (ratio=45.75±2.96 and 33.48±2.56, respectively, amplitude=97.12% and 69.89%, respectively) and at 22.00h for E (ratio=19.33±3.98, amplitude=92.16%). Highest values of the t1/2β ratio were located at 22.00h for B (ratio=1.62±0.15, amplitude=49.48%) at 16.00h for E (ratio=0.86±0.086, amplitude=18.75%) and at 04.00h for M (ratio=1.25±0.04, amplitude=97.75%). Highest values of the AUC ratio were located at 22.00h for B and E (ratio=9.55±0.22 and 23.17±0.71, respectively, amplitude=6.78% and 44.33%, respectively) and at 04.00h for M (ratio=13.60±2.81, amplitude=35.10%). If these results are compared with the acute toxicity data (LD50), it appears that the hour of highest toxicity of B and E well agree with the hour of the highest heart passage, but is in phase opposition for M.

KEY WORDS

Chronokinetics ; heart passage ; local anaesthetics ; toxicity.

INTRODUCTION

Albright (1979), drew attention to a small series of anecdotal reports of cardiac arrest associated with regional anaesthesia and highly lipid-soluble and protein-bound local anaesthetic agents bupivacaine (B), etidocaine (E) and mepivacaine (M) (Edde et al. 1977, Prentis 1979.). This report has lead to a considerable amount of animal experimentation in a effort to clarify the mechanisms by which local anaesthetics, in particular (B), may cause cardiodepression, ventricular arrythmias, cardiac arrest and death, only partly associated with their well described CNS toxicity (Bruguerolle B. and Prat M., 1990). However, no data are available concerning the heart tissue levels following intraperitoneally (IP) administration. Following chronotoxicokinetics studies of B, E and M in mice, it was of interest to document the possible influence of the hour of administration on their respective heart tissue penetration, in order to explain their cardiac toxicity.

MATERIAL AND METHODS

At 10.00h, 16.00h, 22.00h and 04.00h a total of 150 male NMRI mice maintained during the month of october under controlled conditions (LD : 06.00h-18.00h, 18.00h-06.00h, food and

water ad libitum), were given a single IP dose of B (20mg/kg, n=50), E (40mg/kg, n=50) and M (60mg/kg, n=50). Plasma and cardiac tissues were sampled after decapitation 5, 10, 15, 30, 45, 60, 120, 180, 240 and 360 minutes after administration (five animals at each time) and their levels were determined by a GLC method using a flame detection according to Prat and Bruguerolle, (1986). B, E and M serum and cardiac tissue concentration were plotted against time and pharmacokinetic parameters were determined assuming a two compartment open model: maximum concentration (Cmax), ß phase elimination half life (t1/2ß) and the area under the serum and cardiac tissue curve (AUC exp.) were assessed according to conventional methods (Wagner, 1975) by a computer program. The curve fitting of the data was done by the method of residuals according to Wagner. Statistical comparison were done by analysis of variance (ANOVA). The 24hr means were calculated and the temporal changes were expressed by the range.

RESULTS

The cardiac penetration of the local anaesthetics agents (LA) was estimated by the ratio : heart/serum of the three parameters (i.e. Cmax heart/Cmax serum, t1/2ß heart/t1/2ß serum and AUC heart/AUC serum). Table I shows mean±sem values of the ratio of Cmaxheart/Cmaxserum of B, E and M according to the hour of administration. As indicated in this table, a significant difference of the ratio was detected by ANOVA for B, E and M (0.0001, 0.008 and 0.01, respectively).

	10.00h	16.00h	22.00h	04.00h	ANOVA	24hr means	range
B	16.65 ± 2.11	24.76 ± 0.91	32.69 ± 0.78	45.75 ± 2.96	F=33.56 p=0.0001	29.96 ± 5.37	29.10
E	12.55 ± 1.04	17.57 ± 1.38	19.33 ± 3.98	6.45 ± 0.38	F=5.63 p=0.008	13.97 ± 2.5	12.88
M	22.67 ± 2.08	21.22 ± 3.63	17.01 ± 2.5	33.48 ± 2.56	F=5.22 p=0.01	23.59 ± 3.03	16.47
Anova	F=6.25 p=0.01	1.94 0.18	7.56 0.007	61.01 0.0001			

Table I. Mean±sem values of the ratio Cmax heart/Cmax serum
of B, E and M according to the hour of administration
(10.00h, 16.00h, 22.00h and 04.00h) and statistical significancy.

Figure 1 illustrates the circadian variations of the heart penetration of B, E and M. Thus, the higher heart penetration of B and M was observed 04.00h and at 22.00h for E. Concerning the elimination of LA from heart tissues highest values of the t1/2ß ratio were located at 22.00h for B (ratio=1.62±0.15, amplitude=49.48%) at 16 00h for E (ratio=0.86±0.086, amplitude=18.75%) and at 04.00h for M (ratio=1.25±0.04, amplitude=97.75). Concerning the AUC ratio, highest values for the AUC ratio were located at 22.00h for B and E (ratio=9.55±0.22 and 2..17±0.71, respectively, amplitude=6.78% and 44.33%, respectively) and at 04.00h for M (ratio=13.6±2.81, amplitude=35.1%).

Temporal variations of Cmax heart/Cmax serum ratio
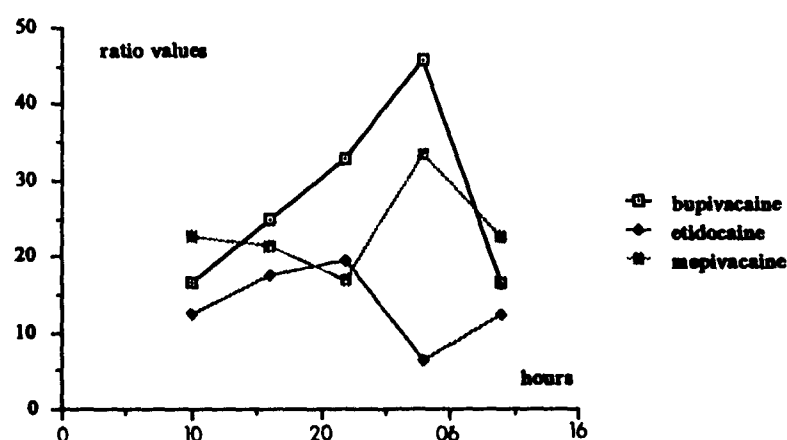
Fig. 1. Temporal variations of Cmaxheart/Cmaxserum ratio

DISCUSSION

Our data indicate a circadian variation of B, E and M heart penetration in mice. Differences in the circadian pattern of heart penetration were demonstrate, as shown in table I (differences in ranges and 24hrs means) these differences in the circadian pattern must be interpreted in terms of differences between these three agents (i.e. differences in physico-chemical properties). Indeed penetration of drugs into heart tissue and between the three LA (as many other biological membranes) depends on their physico-chemical properties such as molecular weight, degree of ionisation, lipid solubility and protein-binding as shown by literature data presented in Table II (Tucker, 1975).

Physico-chemical properties	Bupivacaine	Etidocaine	Mepivacaine
Mol. Weight	288	276	246
pKa	8.1	7.7	7.6
Partition coeff. (lipid solubility)	27.5	141	0.8
Protein-binding	96	94	78

Table II. Physico-chemical properties of B, E and M (data from literature)

As shown in figure 1, the 24hr means of the Cmax ratio (estimating the penetration of the anaesthetic into the heart) are 29.96, 13.97 and 23.59 for B, E and M, respectively. Thus compared to E, the penetration of B and M through the heart tissue is 2 time higher. This better penetration of B and M may be explained by higher lipid solubility for B and the lower molecular weight for M. Finally when these results are compared with acute toxicity (DL50) (Table III) (Prat and Bruguerolle, 1988), (Bruguerolle and Prat, 1990) it appears that the hour of the highest toxicity well agree with the hour of the highest passage in heart (during the dark phase). It also appears from Figure 1 and Table I that the amplitude of the temporal variations is nearly 2 time higher for B, compared to E and M (range : 29.10, 12.88 and 16.47 for B, E and M, respectively.). In conclusion, the temporal pattern of heart passage of the three LA shows a better penetration at the end of the dark phase with different amplitudes explained in part by the circadian variations of lipid solubility and protein binding of these three drugs.

HOURS	10.00h	16.00h	19.00h	22.00h	01.00h	04.00h
B	54.10 ± 2.80	56.40 ± 2.50	54.00 ± 1.30	52.40 ± 0.50	62.00 ± 2.90	56.10 ± 2.80
E	55.00 ± 2.30	52.50 ± 2.10	50.00 ± 1.20	49.00 ± 1.80	49.00 ± 1.40	47.50 ± 2.80
M	130.00 ± 5.00	104.00 ± 6.00	102.00 ± 3.00	110.00 ± 4.00	117.00 ± 3.00	125.00 ± 3.00

Table III. Mean±sem values of the DL50 of bupivacaine, etidocaine and mepivacaine according to the hour of administration (10.00h, 16.00h, 19.00h, 22.00h, 01.00h and 04.00h).

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TEMPORAL VARIATIONS OF BRAIN TISSUE LEVELS OF THE LOCAL ANAESTHETICS IN THE MOUSE

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ABSTRACT

Possible temporal variations in brain tissue levels of three anilide local anaesthetics (LA) : bupivacaine (B), etidocaine (E) and mepivacaine (M) were documented in mice according to the hour of administration (10.00h, 16.00h, 22.00h and 04.00h). Our data indicate a significant temporal change of the brain passage of the three drugs (significant higher value at 10.00h for B and M and 22.00h for E).

KEY WORDS

Brain passage ; chronokinetics ; local anaesthetics ; toxicity.

INTRODUCTION

The systemic toxicity of LA mainly involves the central nervous system (CNS) and the cardiovascular system (De Jong et al., 1977). Toxic and lethal doses of LA drugs usually result in signs of CNS excitation leading to convulsive activity followed by CNS depression and respiratory arrest (Covino et al., 1976). Studies of the relative CNS toxicity of various LA have been carried out in mice (De Jong et al., 1980), rats (Adams et al. 1972), sheep (Morishima et al., 1981), monkeys (Munson et al., 1970) and dogs (Eicholzer, 1976). All these studies involve the administration of LA into extravascular sites or a relatively slow intravenous infusion. However, no data are available concerning the influence of liposolubility and protein binding affinity on the relative CNS toxicity of LA agents followih ; intraperitoneally (IP) administration (this route more closely mimics the clinical situation or an accidental intravascular injection).Following chronotoxicokinetic studies of B, E and M in mice plasma, it was of interest to document the temporal variations of the respective brain tissue penetration of these drugs, in order to explain their respective CNS chronotoxicity.

MATERIAL AND METHODS

Different groups of adult male NMRI mice (n=150), maintained during the month of october under controlled environmental conditions (LD : 06.00h-18.00h, 18.00h-06.00h, food and water were given ad libitum) were injected at four different times of the 24h scale (10.00h, 16.00h, 22.00h, 04.00h) with a single IP dose of B (20mg/kg), E (40mg/kg) and M (60mg/kg). Plasma and brain tissues were collected 5,10, 15, 30, 45, 60,120, 180, 240 and 360 minutes after administration and their levels were determined by a GLC method using a flame detection according to Prat and Bruguerolle, 1986.

The following pharmacokinetic parameters were assessed both in plasma and brain tissue : AUCexp. (µg/ml/h), Cmax (µg/ml) and t1/2ß (h). These pharmacokinetic parameters were determined by conventional methods using a two compartment open-model with first order absorption kinetics using an Apple IIe computer. The curve fitting of the data was done by the method of residuals as described by Wagner (1975). The brain penetration of LA was estimated by the ratio : brain/plasma of these respective three parameters (i.e.Cmax brain/Cmax serum, t1/2ß brain/t1/2ß serum and AUC brain/AUC serum). All data were quantified (mean±sem) and compared by statistical analysis (analysis of variance, ANOVA), the 24hr means were calculated and the amplitude of the temporal change was expressed by the range.

RESULTS

Our results indicate a temporal variation of the brain penetration of LA estimated by the ratio brain/plasma of the three different kinetic parameters. Table I shows mean ±sem values of the ratio : AUCbrain/AUCserum of B, E and M according to the hour of administration. As indicated in this table, a significant difference of this ratio was detected by ANOVA for B and E (p=0.0001 and p=0.003, respectively), but not for M (p=0.98). At each hour of determination the difference between the three LA was significantly detected by ANOVA.

	10.00h	16.00h	22.00h	04.00h	ANOVA	24hr mean	range
B	4.32 ± 0.1	4.3 ± 0.04	2.82 ± 0.11	2.12 ± 0.28	F=51.0 p=0.0001	3.4 ± 0.48	2.2
E	3.89 ± 0.17	3.85 ± 0.11	4.7 ± 0.16	3.93 ± 0.06	F=7.15 p=0.003	4.09 ± 0.17	0.85
M	21.14 ± 3.73	20.78 ± 4.41	19.11 ± 3.02	20.39 ± 2.82	F=0.05 p=0.98	20.39 ± 0.38	2.03
ANOVA	F=14.73 p=0.0008	11.46 0.001	20.80 0.0001	22.85 0.0002			

Table I. Mean±sem values of the ratio AUC brain/AUC
serum of bupivacaine, etidocaine and mepivacaine
according to the hour of administration (10.00h,16.00h,
22.00h and 04.00h) and statistical significancy.

As far as the Cmax brain/Cmax serum ratio is concerned, ANOVA did not revealed a significant variation, the highest value of this ratio being located at 16.00h for B and E (ratio=18.22±1.7 and 22.76±5.2, respectively, amplitude=36.09% and 68.36%, respectively) and at 04.00h for M (ratio=23.22±1.66 amplitude=21.33%). Finally, the elimination of the LA from brain (t1/2ß ratio), is shown in table II (mean±sem values of the ratio : t1/2ß brain/t1/2ß serum). As indicated in this table, a significant variation of the ratio was detected by ANOVA for E and M (p=0.0001 and p=0.03, respectively), and not for B (p=0.17).

	10.00h	16.00h	22.00h	04.00h	ANOVA	24hr mean	range
B	0.69 ± 0.08	0.43 ± 0.09	0.44 ± 0.11	0.29 ± 0.09	F=1.97 p=0.17	0.46 ± 0.07	0.4
E	0.85 ± 0.12	0.99 ± 0.05	3.25 ± 0.42	1.81 ± 0.18	F=17.29 p=0.0001	1.72 ± 0.48	2.4
M	3.26 ± 0.62	1.93 ± 0.7	0.53 ± 0.2	3.06 ± 0.67	F=3.67 p=0.03	2.19 ± 0.54	0.2
ANOVA	F=10.74 p=0.002	2.74 0.16	26.79 0.0001	6.02 0.019			

Table II. Mean±sem values of the ratio $t1/2\beta$ brain/$t1/2\beta$
serum of bupivacaine, etidocaine and mepivacaine
according to the hour of administration (10.00h, 16.00h,
22.00h and 04.00h) and statistical significancy.

DISCUSSION

The aim of this work was first to document possible temporal changes in brain penetration of the three LA, and secondly the relationships with the temporal variations in their acute toxicities. Differences in the circadian pattern of brain penetration, were demonstrated, for the AUC ratio as shown in table I (differences in ranges and 24hr means). These differences in the circadian pattern of brain passage must be interpreted in terms of differences between these three agents (i.e. differences in physicochemical properties). Indeed penetration of drugs through brain tissue depends in part, on their physicochemical properties such as molecular weight, degree of ionisation, lipid solubility and protein binding (Tucker et al. 1975). As shown in table I, the 24hr means of the ratio AUCbrain/AUCserum (estimating the penetration of the drugs through brain tissue during 24.00h) are 3.4, 4.09 and 20.35 for B, E and M respectively : thus compared to B and E, the brain penetration of M is 5 time higher. This better penetration of M in brain tissue may be explained by its lower molecular weight and its lower protein binding (78% of protein binding versus 94% and 96% for E and B, respectively)(Tucker et al. 1975) but do not correlate with differences in lipid solubility of these three drugs, since the better passage and elimination should occur for B and E.
Finally, when these results are compared with acute toxicity (LD50) (Prat and Bruguerolle, 1988), (Bruguerolle and Prat, 1990), it appears that the hour of the highest toxicity of E well agree with the hour of the highest passage in brain as estimated by the Cmax value in brain; at contrary these data do not fit for B and M as shown in table III which illustrates the temporal variations of the rough values of Cmax in brain.

	10.00h	16.00h	22.00h	04.00h	ANOVA	24 hr means
B	7.83 ± 0.84	12.63 ± 3.48	9.80 ± 2.52	6.05 ± 1.21	ns	9.08 ± 1.22
E	18.06 ± 1.94	24.11 ± 3.13	19.74 ± 1.75	25.64 ± 3.83	ns	21.89 ± 1.54
M	239.00 ± 15.86	279.98 ± 39.41	270.50 ± 18.95	333.80 ± 19.92	ns	280.82 ± 17.07

Table III. Mean±sem values of the Cmax value in brain tissue of bupivacaine, etidocaine and mepivacaine according to the hour of administration (10.00h, 16.00h, 22.00h and 04.00h) and statistical significancy.

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CHRONOKINETICS OF ACETAMINOPHEN IN ELDERLY PATIENTS

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ABSTRACT

Possible temporal variations in pharmacokinetic parameters after a single 1 g dose of paracetamol were documented in elderly subjects according to the hour of administration (07.00 h or 19.00 h).Our data indicate in all patients (except one) a different kinetic pattern according to the hour of administration but the group did not reveal any significant temporal changes in kinetic parameters, except for T 1/2 ɑ (significant higher value at 19.00 h, p=0.01).

KEY WORDS

Acetaminophen; chronopharmacokinetics; elderly.

INTRODUCTION

The chronopharmacokinetics of several drugs have been documented and reviewed in animal and in man (Reinberg and Smolensky,1932, Bruguerolle,1987, Lemmer,1981).Most of these studies have documented temporal variations of drug kinetics in healthy and/or young volunteers but very few of them have concerned elderly subjects;we have previously reported chronokinetics of indomethacin (Bruguerolle et al.,1986) or of digoxin (Bruguerolle et al., 1988) in elderly patients. Since temporal variations were demonstrated in normal healthy subjects by Shively and Vesell (1975) or Malan et al.(1985), we decided to investigate possible temporal variations in pharmacokinetic parameters after a single dose of paracetamol in elderly subjects according to the hour of administration.

SUBJECTS AND METHODS

Ten hospitalized male geriatric subjects aged between 70 and 86 years (mean age=76 years) and weighing between 45 and 74 kg participated in this experiment.Our study was a crossover randomized study consisting in a single 1 g oral dose of acetaminophen taken in the morning (07.00 h) or in the evening (19.00 h), each assay being separated by a 5 days wash out period. Meals were taken at 06.30, 11.30 and 18.00 h and subjects were fasting from the evening before (for the morning trial) or from 11.30 h (for the evening trial).Blood samples were taken 0.25, 0.5, 0.45, 1, 1.5, 2, 4, 6, 8 and 12 hours after the drug was administered.Acetaminophen was assayed by fluorescence polarization immunoassay (Abott); the limit of quantitation was 0.7 µg/ml and the coefficient of variation averaged 5 % for concentrations ranging from 0 to 150 µg/ml.Pharmacokinetic parameters were determined in each subjects by conventional methods using a two compartment open-model with first order absorption kinetics using an Apple IIe computer.The curve fitting of the data was done by the method of residuals as described by Wagner (1975) in order to obtain the absorption and elimination rate constants. The elimination half-life (T1/2 ß) was calculated from the elimination rate constant value;

the area under the plasma concentration versus time curve (AUC) was calculated by the trapezoidal rule from time zero to the last point and extrapolated to infinity. Cmax and time to reach it (Tmax) were estimated by inspection.Statistical comparison of the different kinetic parameters was done using the Wilcoxon matched pairs signed ranks test.

RESULTS

The individual and mean plasma levels of acetaminophen determined in elderly subjects after a single 1 g oral dose in the morning or in the evening are shown in figures 1 and 2. The mean±sem pharmacokinetic parameters following the morning or the evening administration and the results of their statistical comparisons are shown in table I.

Our data indicate in all patients (except n° 8) a different kinetic pattern according to the hour of administration (as shown from the individual results in figure 2); at the group (mean±sem) did not reveal any significant temporal changes in kinetic parameters, except for T 1/2 α (significant higher value at 19.00 h, p=0.01).

Hours	Cmax.	T max.	T 1/2 a	T 1/2 α	T 1/2 ß	AUC
07.00 h	27.8 ± 1.51	0.88 ± 0.12	0.31 ± 0.06	0.73 ± 0.14	7.70 ± 1.84	118.8 ± 10.4
19.00 h	23.01 ± 2.62	0.97 ± 0.34	0.39 ± 0.04	1.34 ± 0.22	8.31 ± 1.26	103.9 ± 14.8
Wilcoxon test	T=18 ns	T=10 ns	T=7.5 ns	T=3 p=0.01	T=23 ns	T=13 ns

Table 1.Pharmacokinetic parameters in ten elderly patients according to the hour of administration (07.00 h or 19.00 h); Cmax (µg/ml), Tmax (h),T1/2 a, a and ß (h), AUC (µg/ml/h).

(µg/ml)
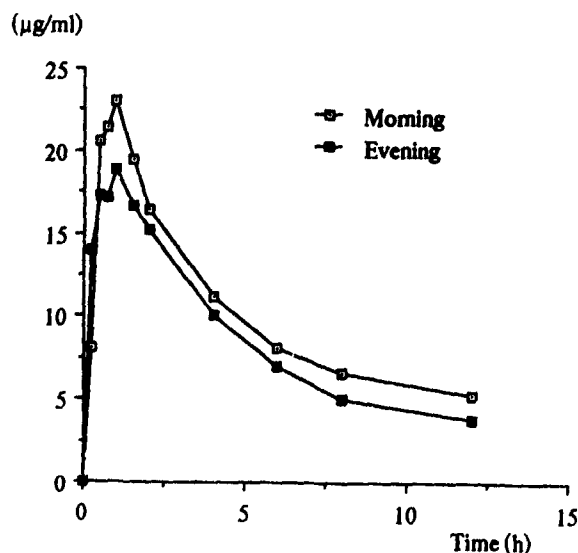
Fig. 1. Mean plasma levels of acetaminophen in elderly subjects.

Fig.2. Individual plasma levels of acetaminophen in elderly according to the hour of administration.
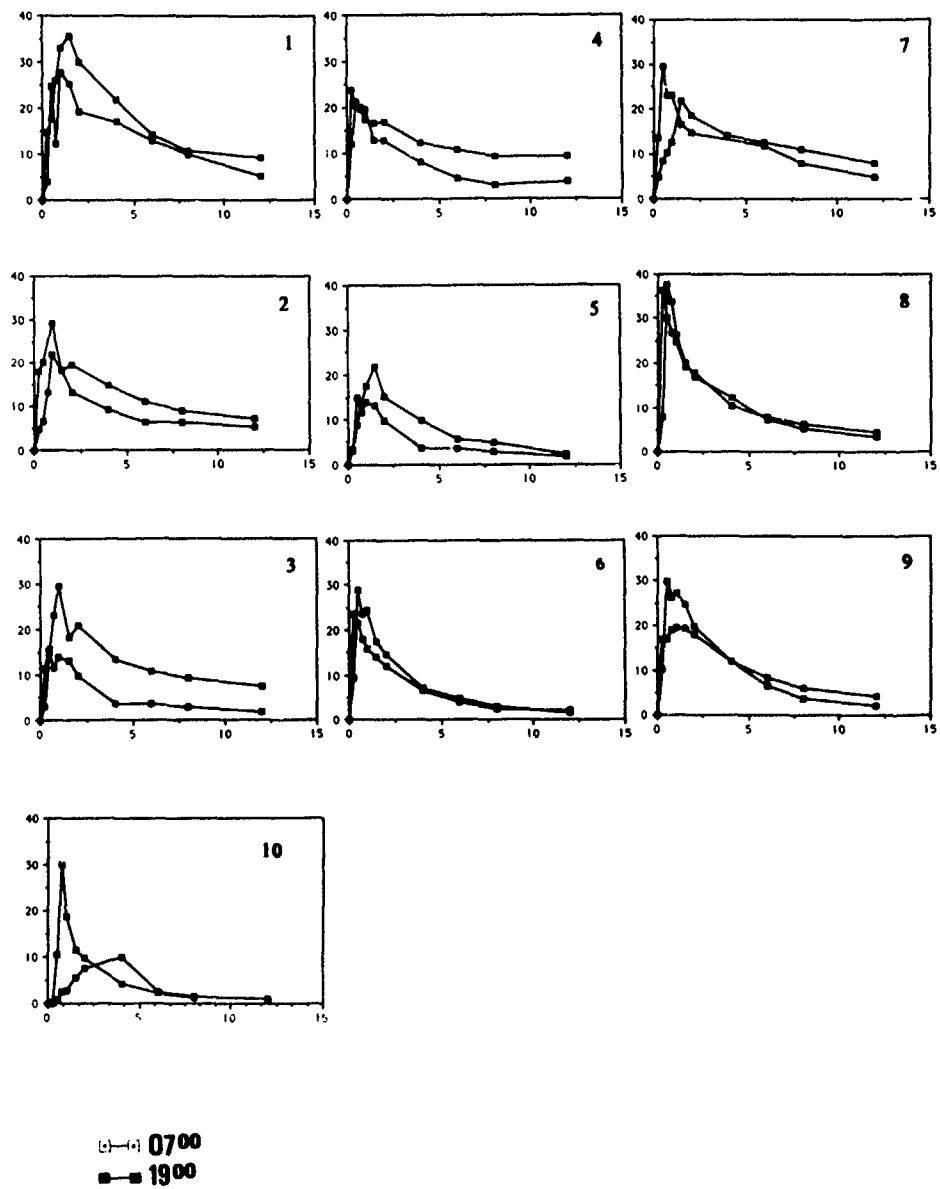
DISCUSSION

Temporal variations in toxicity and kinetics of acetaminophen have been reported in animal and in man. Schnell et al. (1984) reported that mice exhibit a circadian rhythm in acetaminophen lethality (peak at 18.00 h) and an inverse rhythm in hepatic glutathione concentrations: these studies have shown that both lighting schedules and feeding can alter the circadian rhythms in acetaminophen mortality and hepatic glutathione levels in mice.As far as kinetics are concerned, chronokinetics of acetaminophen have been reported in animal (Belanger et al,1987) and in man (Schively and Vesell, 1975, Malan et al., 1985).Thus, in the study of Schively and Vesell, the mean plasma half-lives and volume of distribution were approximately 15 % longer and 13 % lower, respectively, in young adults volunteers at 06.00 h than at 14.00 h ; these results well agree with data from Belanger et al. (1987), acetaminophen metabolism being greater during the activity period in rats. At contrary, Malan et al. (1985) did not found any significant differences in man concerning acetaminophen kinetics in young adults.

Our data in elderly subjects did not demonstrate significant temporal differences in acetaminophen kinetics and in this way well agree with previous reported data of Malan et al.(1985) from a chronopharmacological point of view. Nevertheless, the present study suggest that some pharmacokinetic parameters are modified by the age of the patients. For instance compared to the data of Malan et al.(1985) in healthy young adults, our results reveal that Cmax., AUC and T 1/2 B values are approximately 3 times higher in the elderly. These results agree with previous reported data on acetaminophen kinetics in elderly indicating for instance an increased elimination half-life in elderly subjects (Triggs et al. 1975).

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CIRCADIAN RHYTHM PARAMETERS OF SEVENTEEN HORMONAL VARIABLES IN PLASMA AND OF URINARY CATECHOLAMINES IN ELDERLY SUBJECTS TREATED WITH CYCLOBARBITAL

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ABSTRACT

The circadian variation in 17 hormonal variables was studied in 6 elderly
patients (77 ± 8 years of age) receiving 200 mg cyclobarbital at bedtime. Nine
circadian profiles of 6 plasma and urine samples were collected at 4 hour
intervals over a 24-hour span. The circadian rhythm parameters of the
variables studied were compared with those of 55 subjects of the same age (73,
24-hour profiles) receiving no drug therapy of any kind. A 1 hr 44 minute
phase delay in the circadian rhythm in plasma cortisol in the subjects treated
with cyclobarbital was the only change in circadian timing encountered.

A higher circadian mesor was found in the cyclobarbital treated subjects in
plasma TSH, FSH in women and in urinary epinephrine. A lower circadian mesor
was observed in cortisol, total T4 and C-peptide. The circadian rhythm
parameters of the other variables remained unchanged.

KEY WORDS

Circadian rhythms, elderly, plasma hormones, urinary epinephrine, cyclobarbital

INTRODUCTION

In the application of chronobiology to clinical medicine and particularly to
gerontologic practice, patients frequently receive pharmacologic agents known
to act either on CNS centers thought to regulate circadian rhythms or to
induce enzymes known to alter metabolic parameters showing circadian
periodicity. Some of these agents have been shown experimentally to alter
circadian parameters of clinical interest (Heinemeyer et al., 1986; Zhiri et
al., 1986; Turek and Van Reeth, 1989). The question thus has to be raised as
to what extent circadian reference values obtained from clinically healthy
subjects not receiving any drug therapy are relevant to a gerontologic
population living on the same routine, receiving medication for chronic
conditions.

Frequently elderly subjects suffer from sleep disturbances requiring treatment
with barbiturates. Potential rhythm alterations in barbiturate treated
subjects, i.e., of amplitude and acrophase would have implications for the
evaluation of laboratory measurements of variables showing high amplitude

circadian rhythms and may aid in the design of chronopharmacologic treatment schedules. We are reporting here on a group of elderly subjects receiving cyclobarbital only.

SUBJECTS AND METHODS

Elderly men and women 77 $\pm$ 8 years of age on a diurnal activity pattern (average time of rising 06:00 and retiring 21:00) were studied over one or more 24 hour spans. All subjects followed a diurnal activity pattern with three daily meals at 08:30, 13:00 and 18:00 and with rest at night approximately from 21:00 to 06:00 local time. The subjects were ambulatory before and during the study. Although the time of rising and retiring was to some extent variable it was strongly influenced by the institutional routine. The subjects were studied over one or several 24 hour spans with 6 blood and urine samples obtained at four hour intervals. Seventeen hormonal variables (ACTH, aldosterone, cortisol, C-peptide, DHEA-S, estradiol, FSH, GH, insulin, LH, progesterone, prolactin, 17-OH progesterone, testosterone, TT_3, TT_4, and TSH) were studied by radioimmunoassay in plasma and epinephrine, norepinephrine, and dopamine by high pressure liquid chromatography in the urine. The methodology was reported previously (Haus et al., 1988b). The data were analyzed by single and population mean cosinor (Nelson et al., 1979) and differences in rhythm parameters were verified by the Bingham test (Bingham et al., 1982). Fifty-five subjects (73 profiles) received no drug treatment, six subjects suffering from mild sleep disturbances received 200 mg/day of cyclobarbital at bedtime only, and were studied in 9, 24-hour profiles. Circadian rhythm parameters of subjects receiving only cyclobarbital were compared with those subjects receiving no medications at all.

RESULTS AND DISCUSSION

The variables showing differences in circadian rhythm parameters between subjects receiving cyclobarbital as only medication and subjects receiving no medication of any kind are shown in Table 1. The table indicates the treatment received as no cyclobarbital (No) or cyclobarbital (Cy), with the p-values of the cosinor analysis and the mesor, amplitude and acrophase obtained by population mean cosinor. In instances where cosinor analysis did not allow rhythm detection, only the mesors were compared. Since sampling extended over a full 24 hour period, at equal intervals, the mesor by cosinor is similar to the arithmetic mean. The tentative amplitude and acrophase in instances without statistical rhythm detection provide some insight into the variation of the data over a 24 hour span, but must not be misinterpreted as actual rhythm parameters, and can, therefore, not be used for comparison. Differences in mesor (or arithmetic mean), amplitude or acrophase, statistically verified by the Bingham test, are marked by an asterisk.

As shown in Table 1, the only change in circadian acrophase was found in cortisol with a phase delay of 1 hr 44 minutes in the subjects receiving cyclobarbital. An increase in amplitude was found in cyclobarbital treated women in testosterone. Most differences were in the circadian mean or mesor. In the cyclobarbital treated subjects, there was a decrease in the circadian mesor of cortisol, C-peptide and total T_4, and an increase in TSH and urinary epinephrine. In the women, there was also an increase in FSH (but not in LH). There was a decrease in T_4 and concomitant rise in TSH (Fig. 1) suggesting a lower level of thyroid activity in the cyclobarbital treated patients, and corresponding rise in thyrotropic activity of the pituitary.

Table 1. Changes in circadian rhythm parameters in elderly subjects receiving 200 mg cyclobarbital at bedtime as compared to subjects of comparable age receiving no drug treatment of any kind.

Variable	Sex	Rx	p	Mesor		Amplitude			Acrophase		
				M	± SE	A	95%CI		ø	95%CI	
Cortisol	M+F	No	<0.001	9.32	0.28 *	4.02	3.59	4.45	07:20	06:52	07:48 *
		Cy	0.001	7.55	0.47	3.39	2.24	4.55	09:04	07:24	10:52
C-peptide	M+F	No	<0.001	3.19	0.21 *	1.68	1.37	1.99	16:04	15:20	16:44
		Cy	0.003	1.99	0.39	1.55	0.98	2.13	16:04	14:24	18:32
FSH	F	No	0.544	91.38	5.09 *	0.74			10:12		
		Cy	0.971	115.66	7.36	0.72			21:24		
Prolactin	M+F	No	<0.001	17.15	1.02	4.07	2.64	5.50	01:48	00:44	02:36
		Cy	0.004	20.72	3.26	3.61	1.07	6.15	01:20	23:28	05:48
Testosterone	F	No	0.004	0.44	0.04	0.04	0.02	0.06 *	10:12	07:44	13:04
		Cy	0.019	0.49	0.11	0.13	0.03	0.22	10:36	06:48	12:04
Total T4	M+F	No	<0.001	7.11	0.23 *	0.27	0.18	0.36	12:48	11:40	14:08
		Cy	0.114	5.75	0.51	0.23			12:16		
TSH	M+F	No	<0.001	4.06	0.30 *	0.56	0.38	0.73	01:12	00:40	01:56
		Cy	0.175	6.06	1.85	0.75			01:32		
Ur Epi	M+F	No	0.008	38.95	7.28 *	29.44	10.90	47.99	13:44	12:04	14:56
		Cy	0.471	116.36	62.13	69.03			16:52		

* = difference Cyclobarbital (Cy) vs No Rx (No), $p < 0.05$ in Bingham test.
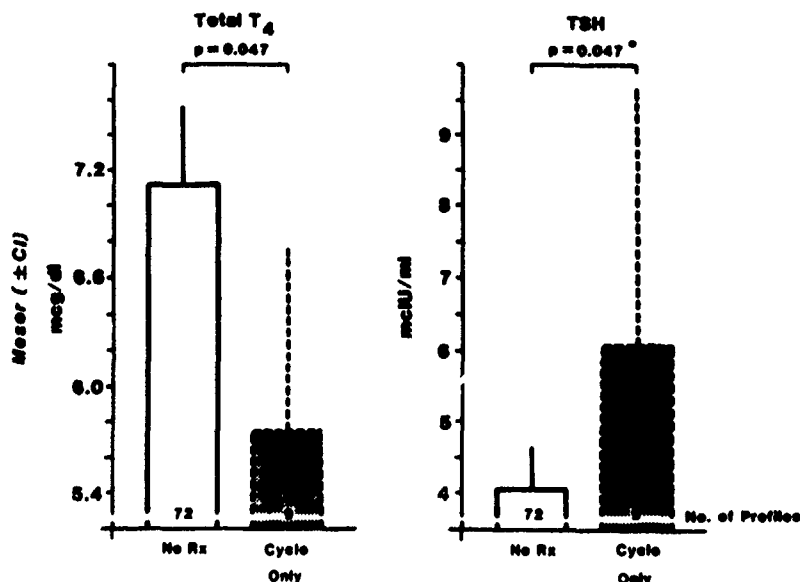
Fig. 1 Lowered plasma total T_4 and concomitantly elevated TSH in elderly patients receiving 200 mg cyclobarbital at bedtime as compared to subjects of comparable age receiving no drug treatment of any kind.

The delay in circadian acrophase in cortisol may be an expression of the
therapeutic effect of cyclobarbital with improved morning sleep. The decrease
in the circadian mesor of plasma cortisol in the treated patients may be the
result of changes in cortisol metabolizing enzyme induction in the liver by
the barbiturate (Park, 1981). Changes in corticosteroid metabolism, with
increased secretion of some metabolites, has been reported in patients
receiving antiepileptic phenobarbital medications (Zhiri et al., 1986).
Induction of enzymes active in drug and hormone metabolism in the liver may be
an important factor leading to changes in biologic activity and clearance
(Heinemeyer et al., 1986).

The increase in circadian mesor in urinary epinephrine excretion has been
found with several barbiturates and barbiturate combinations, meprobamate and
phenothiazines (Haus et al., 1988a). It is of interest that there were no
changes in circadian rhythm parameters of prolactin in the cyclobarbital
treated subjects.

The present study shows that in elderly subjects treated for mild sleep
disturbances, with conventional doses of cyclobarbital, the minor changes in
circadian timing found in plasma cortisol only seem to parallel the
therapeutic effect of the drug. No changes in circadian acrophases were
detected statistically by Bingham test in other functions. Changes in the
circadian mean or mesor of several variables in the cyclobarbital treated
subjects are of interest, but may in some be a manifestation of the underlying
disorder (e.g. urinary epinephrine).

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D-XYLOSE PHARMACOKINETICS DURING THE MENSTRUAL CYCLE

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ABSTRACT

The effects of the menstrual cycle on D-xylose disposition was evalu-
ated in thirteen healthy women normal cycling. Experiments were car-
ried out over two consecutive menstrual cycles during the summer.
This study has demonstrated that the menstrual cycle does not affect
significantly D-xylose absorption. However, our results show a sig-
nificant increase in D-xylose total and renal clearances during luteal
phase of the menstrual cycle. These results are consistent with those
of others showing an increased glomerular filtration rate (GFR) during
luteal phase. Possible mechanisms include the effect of progesterone,
estradiol, or both on GFR.

KEYWORDS

Menstrual cycle; D-xylose; pharmacokinetics

INTRODUCTION

It is well known that gender is a common and obvious difference among
subjects that might potentially affect drugs pharmacokinetic. However,
the influence of the menstrual cycle on drugs disposition is not well
documented, even if among a number of factors which might be expected
to contribute to a gender-related differences in drugs pharmacokinetic,
the most important factors are thought to be the numerous physiological
changes associated to the cyclic variations in circulating hormone lev-
els that occur during the menstrual cycle. Supporting evidence for
this hypothesis has come from a number of studies (Bruguerolle, 1986;
Wilson, 1984)

D-xylose was chosen as a marker for hydrosoluble drugs absorption be-
cause it has been used for many years in assessing gastrointestinal
absorption in clinical practice.

The purpose of this study was to characterize the influence of the
menstrual cycle on the absorption and the elimination of D-xylose in
healthy female volunteers.

MATERIALS AND METHODS

After four-cycle observatory periods of the body basal temperature, 13 healthy women (aged between 18 and 30 years old and normal weighted) have a regular menstrual cycles of 28 ± 2 days received D-xylose during two consecutive menstrual cycles. Each menstrual cycle was divided in three phases: Follicular, ovulatory and luteal phases. Characterized by low levels of gonadal hormone activity, the early follicular phase was chosen (days 2-7). Volunteers were also tested near ovulation when surges of luteinizing hormones (LH), follicle-stimulating hormone (FSH), and estradiol(E_2) occur; ovulation time was characterized by LH levels and body basal temperature. Indeed, further confirmation of the ovulation was obtained by progesterone and estradiol dosages. Associated with relatively high concentrations of progesterone and estradiol the midluteal phase was also selected (days 20-25). Two consecutive days during each menstrual cycle phase, the subjects received: The first day, oral doses of D-xylose, as 25 g in 200 mL of water, and the second day, 5 g of D-xylose in 50 mL of normal saline was infused intravenously over two minutes. The same study design was repeated over the next menstrual cycle. This study was carried out from June 1988 to September 1988. Blood and urine samples were obtained at the same intervals under the same conditions after oral and intravenous administrations. Blood samples were drawn from an indwelling catheter in antecubital vein to the following sequence: 0, 5, 10, 15, 20, 30, 45 min and 1, 2, 3, 4, 5, 6, 7 and 8 hours after dosing. Urine was collected immediately before administration of D-xylose and at intervals between 0 and 2, 2 and 4, 4 and 6 and 6 and 8 hours after dosing. D-xylose was measured in plasma and urine with a phloroglucinol-based colorimetric method (Hebert et al, 1979, Johnson et al, 1984). Pharmacokinetic parameters were estimated by compartmental analysis, with and extended least squares regression program: MK MODEL 3.13. Analysis of variance for repeated measures was used for the statistical analysis.

RESULTS AND DISCUSSION

Results from the present investigation reveal that significant variations occured in drugs disposition throughout menstrual cycle. Tables 1 and 2 show the more relevant results of this study. The hormonal changes across the menstrual cycle does not affect D-xylose absorption. Although, D-xylose peak plasma concentrations (C_{max}) following oral administration were decreased during luteal phase of Cycle 1 by 18% and 19% comparatively to follicular and ovulatory phases, respectively: this was an isolated finding which does not repeat itself in the Cycle 2 and was not accompanied by other significant changes in D-xylose absorption.

This study indicates that D-xylose is cleared from the body faster during luteal phase than the other phases of the menstrual cycle. D-xylose total and renal clearances were increased by 8% to 25% during luteal phase following oral and I.V. administrations (Tables 1 and 2). Our results support the hypothesis that hormonal changes seen during the menstrual cycle affect renal elimination. Previous reports have demonstrated a 5-20% increase in GFR during luteal phase, which is consistent with our results (Brøchner et al 1987; Davison and Noble, 1981; Paaby et al, 1987; Bisdee et al, 1989). The most likely explanation appears to be related to the effects of progesterone or estradiol, or both on GFR.

It appears that sex hormones play a role in gender differences in drug disposition. Then, it is obvious that the experimental design of investigations should considered the phases of the menstrual cycle in female subjects. Further pharmacokinetic studies should regard the menstrual cycle as a potential source of variability.

Table 1. D-xylose Pharmacokinetic Parameters Oral
Administration

	Follicular	Ovulatory	Luteal
Cycle 1			
Cmax (μg/mL)	484 ± 145	485 ± 95	409 ± 114*
CL (mL/min/Kg)	2.76 ± 0.53	2.75 ± 0.46	2.94 ± 0.43
CL_R (mL/min/Kg)	1.05 ± 0.35	1.25 ± 0.32	1.28 ± 0.41**
$T_{1/2 \beta}$ (H)	0.98 ± 0.16	1.06 ± 0.34	1.05 ± 0.18
Cycle 2			
Cmax (μg/mL)	419 ± 104	374 ± 84	375 ± 56
CL (mL/min/Kg)	3.36 ± 0.92	3.23 ± 0.61	3.91 ± 0.92*
CL_R(mL/min/Kg)	1.44 ± 0.41	1.50 ± 0.28	1.63 ± 0.30
$T_{1/2 \beta}$ (H)	1.13 ± 0.22	1.04 ± 0.17	0.87 ± 0.18*

Table 2. D-xylose Pharmacokinetic Parameters
IV Administration

	Follicular	Ovulatory	Luteal
Cycle 1			
CL (mL/min/Kg)	2.43 ± 0.39	2.52 ± 0.39	2.74 ± 0.47*
CL_R (mL/min/Kg)	1.05 ± 0.37	1.04 ± 0.51	1.38 ± 0.31*
$T_{1/2 \beta}$ (H)	1.09 ± 0.15	1.08 ± 0.12	1.02 ± 0.12
Cycle 2			
CL (mL/min/Kg)	3.00 ± 0.68	2.98 ± 0.54	3.45 ± 0.75*
CL_R (mL/min/Kg)	1.47 ± 0.47	1.39 ± 0.26	1.58 ± 0.45*
$T_{1/2 \beta}$ (H)	1.07 ± 0.12	1.01 ± 0.13	1.00 ± 0.15

*$p < 0.05$ (ANOVA, repeated measures); ** $\bar{p} = 0.08$

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CHRONOPHARMACOKINETICS OF GENTAMICIN: COMPARISON BETWEEN MAN AND MICE

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ABSTRACT

The present work was performed to clarify the effects of the dosing time of the day on the pharmacokinetics of gentamicin in man and mice . The significant circadian rhythm was demonstrated in gentamicin kinetics , in both man and mice , showing the lower clearance (CL) in the rest period and the higher CL in the active period . The rhythm seems to well correlate to the subchronic toxicity of the drug .

KEY WORDS

Circadian rhythm ; chronopharmacokinetics ; gentamicin ; clearance ; toxicity ; man ; mice .

INTRODUCTION

The circadian rhythm of susceptibility to gentamicin was first reported in mice by our group (Nakano et al , 1982) . The following investigation confirmed this finding , indicating the circadian rhythm in the susceptibility of mice to other aminoglycosides , and showed great similarity in their chronotoxicity (Soulban et al , 1986 ; Pariat et al , 1984 ; Fisch et al , 1984 ; Pariat et al , 1986) . The circadian stage - dependent changes in gentamicin kinetics has been reported in rats (Lakatua et al , 1986) . The present work was performed to clarify the effects of the time - of - day of drug administration on the pharmacokinetics of gentamicin in man and mice and the relationship between the chronopharmacokinetics and the subchronic toxicity of the drug in mice .

METHODS

Human study

Nine healthy male Japanese medical students participated in the study . Subject took gentamicin , 80 mg / body , i . m ., on three occasions (0800 , 1600 or 0000) , using latin square design . Subjects kept the regular life with diurnal activity and nocturnal rest . Plasma samples were drawn before and 0.5 , 1 , 1.5 , 2 , 3 , 4 , 6 and 8 hr after injection .

Animal study

60 male ICR mice were housed in a standardized light - dark (LD) cycle (LD 12 : 12 ; L : 0700 - 1900) with food and water ad libitum . A single dose of gentamicin 10 mg / kg , s . c . was used   Mice were given the drug at one of six times : 0900 , 1300 , 1700 , 2100 , 0100 and 0500 . Multiple plasma samples (approximately 60 µl for each sample) were drawn by orbital sinus collection by micropipets at 0.5 , 1 , 2 , 3 and 4 hr after injection .

40 male ICR mice were used for the subchronic toxicity study . Groups of 20 animals each received the drug once daily at the midlight (1300) or at the middark (0100) . Each animal was given gentamicin 220 mg / kg / day , s . c . for 18 days . Mortality rate was measured at the same clock - hour every day .

Assay of plasma gentamicin concentrations

Total gentamicin concentrations in plasma were measured by homogenous enzyme immunoassay (EMIT system) . The coefficient of variation of identical samples was less than 10 percent . One - compartment open model was employed to calculate kinetic parameters : elimination rate constant (Ke) , elimination half - life (t 1 / 2) , volume of distribution (Vd) , total body clearance (CL) and area under the curve (AUC) .

Statistical evaluations

Statistical evaluation was performed by analysis of variance (ANOVA) and Tukey's test was used to determine the significance of clock - hour difference between different dosing times .   The generalized Wilcoxon test was employed for statistical evaluation of the toxicity data .

RESULTS

Human study

A significant circadian rhythm was demonstrated for gentamicin kinetics with the lowest CL , the longer t 1 / 2 and the larger AUC at the midnight (Table 1) .

Animal study

A highly significant circadian rhythm was demonstrated for gentamicin kinetics with the lowest CL , the longer t 1 / 2 and the larger AUC , at the midlight .

Table 1

Chronopharmacokinetics of Gentamicin in Man (80 mg, i m)

	Time of administration			Statistical significance
	Morning (0800)	Evening (1600)	Midnight (0000)	(ANOVA)
Ka (hr^{-1})	5 98 (1 08)	4.42 (0 47)	5 65 (0 77)	N S
Cmax (µg/ml)	6 84 (0.24)	6 78 (0 44)	6 58 (0 32)	N S
tmax (hr)	0 37 (0.05)	0 42 (0 03)	0 39 (0 05)	N S
AUC$_0^\infty$ (µg hr/ml)	17.3 (0 8)	15 5 (2 0)	19 0 (0.7)	P = 0 02
Vd (l)	9.97 (0 43)	10 03 (0.59)	10 58 (0 51)	N . S
Ke (hr^{-1})	0.477 (0 026)	0 475 (0.037)	0 408 (0.023)	P = 0 08
t½ (hr)	1 49 (0 08)	1 52 (0 11)	1 75 (0.10)	P = 0 03
CL (l/hr)	4 71 (0 25)	4 60 (0 12)	4 26 (0.16)	P = 0 04

N = 9 , Mean ± (SE)

There was a significant dosing - time dependency in the subchronic toxicity of the drug in mice . The mortality was significantly higher in mice injected at the midlight than at the middark (p = 0.04) .

DISCUSSION

In the present study , a significant circadian rhythm of gentamicin kinetics was demonstrated in man and mice . The significant circadian rhythm in gentamicin kinetics showed the lower CL in the rest period and the higher CL in the active period , in both man and mice . The exact mechanism developing the temporal variations in gentamicin kinetics is not known at the present . It is known that gentamicin is excreted largely unchanged in the urine . The circadian rhythm in glomerular filtration rate is known to show the maximum value at the middark corresponding to the time - point with the highest clearance of the drug .

The finding of subchronic chronotoxicity in mice well coincided with the rhythm in the gentamicin kinetics . Thus the results suggest that the timing of dosing is critically important to prevent the toxicity in drug therapy with gentamicin .

CONCLUSION

The significant circadian rhythm was demonstrated in gentamicin kinetics , in both man and mice , showing, the lower CL in the rest period and the higher CL in the active period . The rhythm of pharmacokinetics of the drug seems to well correlate to the subchronic toxicity of the drug in mice .

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PLASMA CONCENTRATIONS OF METHYLXANTHINES UNDER CIRCADIAN THERAPY WITH THEOPHYLLINE

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ABSTRACT

Toxic side effects may occur under theophylline therapy even in patients whose serum theophylline concentrations are well in the therapeutic range. Caffeine, contained in beverages and drugs, has similiar desired and undesired effects as theophylline. The consumption of caffeinated beverages varies largely among individuals and even in one and the same person. The circadian variation in plasma caffeine concentrations was therefore studied in nine male asthmatic patients before and under a circadian theophylline therapy (Bronchoretard*, 350 mg at 08h00, 700 mg at 22h00). Theophylline concentrations were in the therapeutic range and varied between 4.9 µg/ml (22h00) and 16.4 µg/ml (14h00). Caffeine concentrations ranged from undetectable (06h00) to 6.4 µg/ml (14h00). Interindividual variation of caffeine plasma concentrations was highest in the early afternoon (14h00) and lowest in the early morning (06h00). The results demonstrate that both theophylline as well as caffeine plasma concentrations should be monitored to control the therapeutic range of a theophylline therapy for bronchial asthma. Drug monitoring is suggested two times per 24 hours, right after awakening of the p ient (at 08h00) and at 14h00.

KEYWORDS

Theophylline, caffeine, drug monitoring, plasma concentrations

INTRODUCTION

Caffeine (in food and drugs) has similiar desired and undesired effects. It may be unconsciously coadministered with theophylline, thereby adding to the drug's toxicity. The main source of caffeine are certainly caffeinated beverages, such as coffee, tea, cola etc.. The caffeine dose per cup of coffee differs in the literature between 60 mg per cup (Bonati and Garattini, 1988) and 155 mg (Zylber-Katz et al., 1984). Caffeine is also a component of some drugs, e.g. analgetics. Theophylline is administered today mainly as retarded tablets with a time to peak plasma concentration of 4 - 6 h. Caffeine absorption from caffeinated beverages is quick and complete: the time to reach maximal plasma concentrations is about 50 minutes (Abernathy and Todd, 1985, Beach et al., 1986). The elimination half-live of caffeine and theophylline ranges between 4 to 6 h in nonsmoking healthy subjects (Scott et al., 1988 Mays et al., 1987, von Borstel, 1983).
The determination of plasma drug concentrations ("drug monitoring") has become a valuable tool for controlling the administration of drugs witn low therapeutic range such as theophylline. To assess the relevance of monitoring all possible methylxanthines under theophylline therapy we were interested in the individual variation of the plasma concentrations of

caffeine and theophylline within 24 hours.

MATERIALS AND METHODS

The plasma concentration of both methylxanthines was determined in nine male asthmatic patients (19–39 years of age). Venous blood was drawn at 4h intervals for 24 hours before and on day 7 of a circadian therapy with 1050 mg/d theophylline (BronchoretardR, 350 mg at 08h00, 700 mg at 22h00). The subjects were hospitalized for the duration of the study; they did not stay in bed during the day and were allowed to go for short walks; lights were turned out from 23h00 – 07h00 and meals were served at 12h00, 19h00, and 08h00. The patients were asked to continue their normal consumption of caffeinated beverages and record time and number of cups drunken.

Blood samples were centrifuged immediatly after collection and the plasma was stored at –20° C till the assay. Plasmaconcentrations of theophylline (TP) and caffeine (CA) were determinded by means of high pressure liquid chromatography (HPLC). Analysis was performed using a reversed phase column (ODS-Hypersil 3µ, 125x4,1mm) eluted with a phosphate–buffer � isopropanol (97:3, v/v, pH 7,0) at a flow rate of 0.5 ml/min. The HPLC system consisted of a constant flow pump (model 600/200, Gynkothek München, Germany) and a variable wavelength spectrophotometer (model SP-4, Gynkothek München, Germany) set at 275 nm. The lower limit of detection was 0.10 µg/ml plasma for both substances.

140 µl of the plasma were deproteinized with 15 µl 0.6n perchloric acid and twice extracted in 2 ml chloroform–isopropanol (85:15, v/v). The organic phase was removed, evaporated under a stream of nitrogen at 45° C, and resuspended in Aq. bidest.. 20 µl of this solution was injected into the HPLC system.

RESULTS

The consumption of caffeinated beverages varied largely. One patient drank 1 cup of coffee on the day before and 6 cups on day 7 of theophylline therapy (with the respective increase in plasma caffeine concentrations, Fig. 1), another one changed his coffee consumption from 3 to 0 cups. Both and a third person who did not comply to the prescribed theophylline dosing regimen were excluded from the final group analysis. The coffee consumption of the remaining 6 patients was 1.4 ± 0.6 (mean ± SD) cups of coffee per day and per person on both days of the investigation.

Fig. 1:   MALE ASTHMATIC PATIENT (F.)
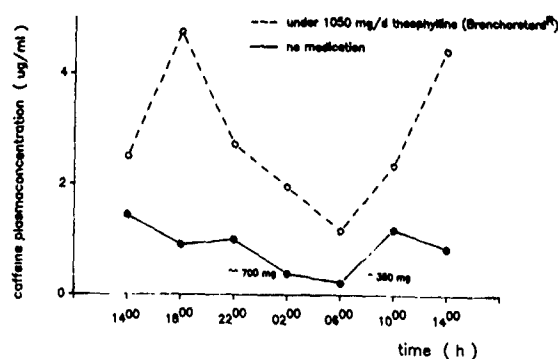
Before theophylline treatment the CA 24h–mean ± SE was 0.8 ± 0.3 µg/ml with a circadian range from 0.4 µg/ml (06h00) to 1.1 µg/ml (14h00). The individual values varied from undetectable to 6.2 µg/ml.

Under treatment with theophylline TP concentrations were in the lower therapeutic range with a 24–mean ± SE of 8.8 ± 0.5 µg/ml and a circadian range of 6.7 µg/ml (22h00) to 10.2 µg/ml (10h00, Fig. 2). The individual values varied between 4.9 µg/ml (22h00) and 16.4 µg/ml (14h00). Caffeine concentrations increased to 1.3 ± 0.4 µg/ml (24–mean ± SE) with a

circadian range 0.6 µg/ml (06h00) - 2.2 µg/ml (18h00, Fig. 2), individual concentrations ranged from undetectable (06h00) to 6.4 µg/ml (14h00).

Fig. 2   MALE ASTHMATIC PATIENTS UNDER CIRCADIAN THEOPHYLLINE THERAPY
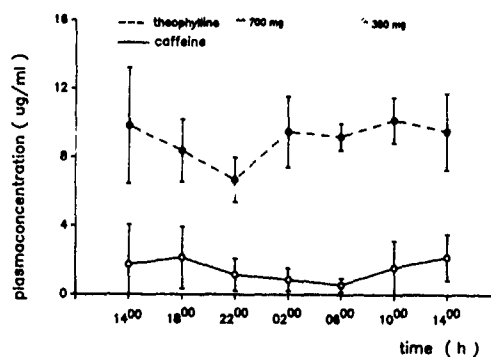
The sum of TP and CA concentrations amounted to 10.1 ± 1.0 µg/ml (24-mean ± SE) with a circadian range of 7.3 µg/ml (22h00) - 11.5 µg/ml (10h00), individual values ranged from 5.2 µg/ml (22h00) to 22.9 µg/ml (14h00, Fig. 3). Interindividual variation was largest in the early afternoon.

Fig. 3

MALE ASTHMATIC PATIENTS
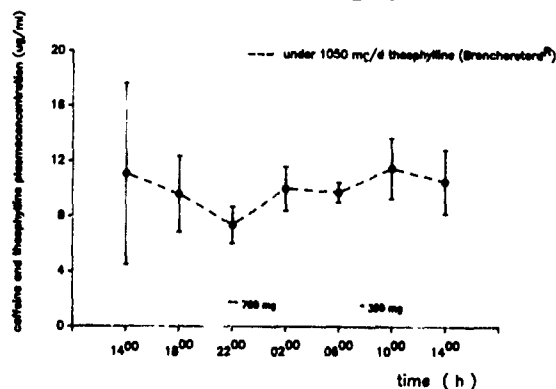
DISCUSSION

The TP plasmaconcentrations detected in this study validate the circadian dosing regimen (one third of the daily dose at 08h00, two thirds at 22h00). By this way fairly stable plasma theophylline concentrations are obtained throughout the day with high levels particularly in the early morning hours. The higher dose at night is necessary to counteract the slower absorption at that time of day (Steinijans et al 1986, Haen & Emslander 1989).
The CA concentrations varied largely among the subjects with extreme values of 6 4 µg/ml in patient M and undetectable values in patient L. There are also large intraindividual variations of CA plasma concentrations within 24 hours CA concentrations are particularly

high during daytime indicating food as primary CA source. It is important to note that coffee consumption is not stable anu may change from day to day as indicated by the two of the three patients excluded from the final group analysis. In individual patients this CA consumption may become toxicologically relevant. Particularly in the early afternoon the concentration of CA has to be considered in medical treatme.it with methylxanthines. This implies that all methylxanthines contained in food (caffeine) and drugs (caffeine, pentoxifylline) should be monitored for evaluation of the therapeutic range during longterm treatment with theophylline. We suggest blood specimens to be drawn right after awakening of the patients (at 08h00) and 14h00 for this purpose.

Since caffeine concentrations were higher under theophylline therapy than without the drug a transformation of theophylline to caffeine might be also considered (Tang-Liu & Riegelman 1981). This has been described for children (Aranda et al 1985). However, the increase observed in our study was statistically not significant

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PHASE-ADVANCE OF CIRCADIAN RHYTHMS BY ENVIRONMENTAL CUE MANIPULATION

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ABSTRACT

A protocol of dietary and sleep/wake modifications was used in an attempt to phase-advance the circadian rhythms of several physiological variables. The protocol, originally developed by Ehret, Groh, and Meinert (1981) to preadjust travelers, was modified based on previous studies in our own lab and used in 25 healthy volunteers who were divided into chronotypes using the Morningness-Eveningness Questionnaire (MEQ) (Horne and Ostberg, 1976). Significant phase-advancement was seen in the temperature, systolic and diastolic blood pressure rhythms of evening types, and in the diastolic blood pressure rhythms of neutral types ($p < .05$). Effects seen in the other variables were neither as dramatic nor sustained.

KEYWORDS

circadian, phase-advance, morningness-eveningness, humans

INTRODUCTION

Previous studies have suggested that surgery causes circadian disruptions similar to those associated with jet-lag (Farr et al., 1984). Applications of a protocol of dietary and light/dark cycle modifications have been successful in reducing the circadian timing alterations seen following surgery in rats (Farr et al., 1985; Farr et al., 1987). The purpose of this study was to test the ability of the protocol to phase-advance the circadian rhythms of healthy humans. The study was designed as a preliminary test of the protocol before use in a human surgical population. Application of this protocol in a human sample could possibly lead to increased feelings of well-being and shorter recovery periods.

MATERIALS AND METHODS

Thirty-two healthy human subjects between the ages of 20 and 55 were recruited for the study. Subjects were day-active with no acute health conditions. No subjects took medications know to alter circadian rhythmicity. Two subjects withdrew from the study before protocol completion. Of the 30 subjects who completed the study, seven were dropped from the analysis because of questions of absolute compliance with some part of the protocol. The final sample included 17 females and 7 males with an average age of 34.9 (SD 8.2) years. Each subject completed the MEQ. Scores indicated 11 morning types, 3 evening types, and 9 neither morning nor evening types. Subjects collected data using an

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autorhythmometry procedure modified from the one described by Halberg et al. (1972). Temperature was measured using a BASIS Electronic Digital Thermometer, heart rate and blood pressure were measured using a Digitronic II Blood Pressure device, peak expiratory airflow (PEF) was measured using a Palrod Floscope, and grip strength was measured using a TEC Dynamometer. Mood and vigor were rated on a 7-point scale designed to measure variations in the usual feelings experienced at a given time of day. Urine was collected for sodium and potassium. Subjects were asked to take their initial set of measurements immediately after awakening and every two hours thereafter throughout their awake period. Each subject collected at least eight data points per day for 12 days; four days of baseline data, four days of protocol data, and four days of follow-up data. During the baseline phase, subjects maintained their normal diet and sleep-wake schedule with the exception that they eliminated caffeine from their diet. During the protocol period, days 5, 6, 7, and 8, subjects awakened one hour earlier and retired one hour earlier, drank at least 16 ounces of a caffeinated beverage within the first 90 minutes of their awake period, and ate at least 3 ounces of protein within an hour of awakening. Total caloric intake for these days alternated between 1000 and 1500 calories, and a balance between carbohydrates and proteins were prescribed for the remainder of the day. Each day of protocol, subjects were given a wake-up telephone call to ensure the earlier arising time. During the telephone call, the research nurse reviewed the expected dietary modifications, reinforcing the protein, caffeine, and caloric consumption requirements for the day, and answered any questions the subjects had. During the follow-up interval, subjects resumed their normal diet and sleep-wake habits but again abstained from caffeine.

Table 1. Summary of modifications for diet and sleep-wake cycles.

Baseline Period: Days 1 - 4
Diet: Abstain from caffeinated foods or beverages. Decaffeinated drinks are allowed.
Sleep-Wake: Continue with usual schedule
Protocol Period: Days 5 - 7
Diet: Eat at least 3 ounces of protein within one hour of awakening. Drink at least 16 ounces of a caffeinated beverage within 90 minutes of awakening. Abstain from caffeine the rest of the day. Eat a balance of protein and carbohydrates throughout the day but limit total caloric intake to 1000 kcal.
Sleep-Wake: Arise 1 hour earlier and go to bed 1 hour earlier than your usual schedule.
Protocol Period: Days 6 & 8
Diet: Continue as above; you may increase your calorie intake to 1500 kcal.
Sleep-Wake: Continue awakening and retiring one hour earlier that usual.
Follow-up Period: Days 9 - 12
Diet: Abstain from caffeine foods and beverages. Resume normal eating.
Sleep-Wake: Resume usual sleep patterns.

Data were analyzed using cosinor and time-series serial section analysis. Both single cosinor and group mean cosinor values were calculated. Results presented here represent group mean acrophases calculated for morning, evening, or neither-type subjects during the baseline, protocol and follow-up periods.

RESULTS

Table 2 displays the acrophases in hours for each variable, during each interval of data collection, for each chronotype. Temperature acrophases for evening-types began a phase-advance during the protocol period which became significant during the follow-up period. Morning and neither types did not change significantly. Heart rate acrophases for the three groups remained unchanged.

Systolic blood pressure for evening-types advanced significantly during the protocol interval with a tendency to return to baseline during the follow-up

period. A slight advance from baseline occurred in morning people during
protocol; baseline was resumed during follow-up. Neither-types did not change.

Table 2. Group acrophases in hours for each variable and chronotype
during the study intervals

		Baseline	Protocol	Follow-up
Temperature	AM	17.16	15.60	17.16
	NT	16.48	17.37	16.42
	PM	16.58	15.27	13.35*
Heart Rate	AM	18.28	19.21	18.28
	NT	17.36	18.16	15.23
	PM	18.08	19.30	16.36
Systolic BP	AM	15.46	13.25	15.19
	NT	16.21	15.30	16.34
	PM	20.29	16.05*	17.43
Diastolic BP	AM	9.44	12.06	11.53
	NT	11.10	9.01*	.55*
	PM	23.15	16.48*	9.34*
Mood	AM	13.96	13.96	12.87
	NT	14.43	13.85	11.24
	PM	14.37	15.20	15.95
Vigor	AM	15.26	12.55	13.80
	NT	15.19	13.92	15.11
	PM	15.44	13.48	14.49
Left Grip	AM	12.97	13.78	15.44
	NT	15.85	15.54	15.75
	PM	15.86	16.11	14.88
Right Grip	AM	13.02	14.03	15.20
	NT	16.45	15.37	15.68
	PM	15.41	14.35	15.46
PEF	AM	12.55	12.59	13.74
	NT	13.13	11.73	13.88
	PM	14.54	14.73	15.25
Na/K	AM	2.66	7.66	9.41
	NT	6.69	2.90	5.72
	PM	2.99	1.22	2.61

AM = Morning-type;   NT = Neither-type;   PM = Evening-type
* $p < .05$

Diastolic blood pressure responded more dramatically than other variables.
Evening-type persons demonstrated a major and significant phase-advance during
protocol and continued to advance in the follow-up period. The neither-type
group phase advanced non-significantly during protocol, then demonstrated a
significant and major advance during the follow-up period. Morning-types did
not change.

Acrophases for the remaining variables of mood, vigor, left and right grip
strength, peak expiratory air flow (PEF) and sodium/potassium (Na/K) ratio were

affected in a variable and non-significant manner. There was a tendency for evening and neither types to change in the desired direction.

DISCUSSION

The protocol tested was partially successful in phase-advancing the circadian acrophases of the sample The effectiveness of the protocol appears to be dependent upon the chronotype of the individual. Both significant and non-significant phase-advances tended to occur predominantly in evening or neither-type subjects. Morning subjects were less responsive. There are two possible explanations for this observation. The first possibility assumes that human beings, like other animals, have a "window of sensitivity" within their 24-hour day (Aschoff, 1965a & b). The application of cues such as advancing activity onset and consumption of chronobiotics, such as caffeine and protein foods, will have different effects dependent upon when they are encountered within the circadian cycle. Since morning-type subjects tended to peak earlier than the neutral or evening-types during baseline, it may mean that any cues given to advance the cycle need to be given earlier to morning types. Another possibility which may explain the reduced response of the morning subjects to the chronobiotic protocol lies in their higher degree of rigidity. Hildebrandt and Stratmann (1979) found that evening-type nurses reacted to shiftwork by flattening circadian rhythms while morning-types increased circadian amplitude. Additionally, Colquhoun (1979) found that transmeridian flight caused greater phase-shifting in evening-types than in morning individuals. Folkard et al. (1979) suggests that flexibility of sleep habits as well as other personality factors may influence rhythm adjustments. Further research is needed in order to refine the protocol to enhance its potential for use in all chronotypes.

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SECTION 7

CHRONOTHERAPEUTICS

Chairpersons: M. H. Smolensky and E. Haen

TIMING OPTIMIZES SUSTAINED-RELEASE KETOPROFEN TREATMENT OF OSTEOARTHRITIS

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KEYWORDS

chronotherapy - osteoarthritis - ketoprofen - clinical trial - antiinflammatory agent

INTRODUCTION

Three pharmacokinetic studies (1,2,3) performed with ketoprofen have indicated that its time of administration largely influences its pharmacokinetic parameters. In a first study a single oral dose was given to healthy human volunteers at four different times of day (1). After the morning dose plasma levels of this antiinflammatory agent were higher and attained more quickly. In a second experiment performed in patients, despite a constant intravenous infusion, large circadian changes in plasma levels were observed (2). However using a sustained-release preparation of ketoprofen the circadian changes in pharmacokinetics were minimized (3). Considering these data a clinical chronotherapeutic trial was undertaken. The objective was to evaluate the influence of dosing time of ketoprofen on its tolerance and efficacy.

PATIENTS AND METHODS

A double blind clinical trial was carried out in 118 outpatients with osteoarthritis of the hip or knee. Patients were treated 14 days with an oral preparation of sustained-release ketoprofen (KSR, 200 mg once daily). Patients were randomised in two parallel groups. 59 patients received ketoprofen at 8 am and a placebo of ketoprofen at 8 pm (morning administration of KSR) and 59 patients had the opposite schedule (evening administration of KSR)

Eligible patients were stratified according to the circadian variation of self-rated pain intensity. Tolerance was evaluated by spontaneous recording of adverse effects and by overall assessment by patient. Efficacy was evaluated by visual and verbal pain-scale and by overall assessment by patient and by physician.

RESULTS

. Protocol deviations :

Four patients did not complete the trial. There were two trial dropouts because of intolerance in the morning group, one patient lost from sight and one patient with ischemic stroke in the evening group.

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<u>Comparability of the two groups at time of inclusion</u> :

The two groups were comparable regarding all of the baseline data recorded (physical characteristics, characteristics of osteoarthritis) as well as severity of pain measured initially (Table I).

Table I - Comparability of patients groups at time of inclusion. Validation of the difference between group by Chi Square test.

Baseline Data		Morning administration N = 59	Evening administration N = 59	Chi Square test
Age (years)		63 ± 10	65 ± 9	N.S.
Height (cms)		164 ± 8	162 ± 8	N.S.
Sex	Male	40	41	N.S.
	Female	19	18	N.S.
Osteoarthrisis				
	Knee	33	36	N.S.
	Hip	26	23	N.S.
Visual pain scale (cms)		$67,3 \pm 10$	$67,7 \pm 14$	N.S.

. <u>Comparative tolerance of the two groups</u> :

The tolerance was twice better for the evening group than for the morning one : However, after the evening administration only 11 patients ($\square$19 %) experienced at least one adverse effect against 23 patients ($\square$40%) for the morning administration (Chi Square test : p < 0 01, figure 1). For the evening group, 13 adverse effects were recorded (2 patients experienced 2 adverse effects) against 28 adverse effects (5 patients experienced 2 adverse effects) for the morning group (Student t test $\cdot$ p = 0.03, figure 2). Most of these adverse effects consisted in gastro-intestinal symptoms.
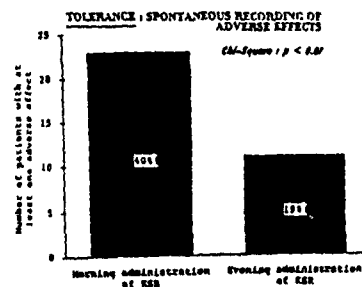
Figure 1
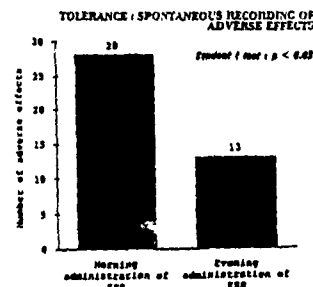
Figure 2

Figure 1 and 2 - Tolerance of Ketoprofen sustained-release (KSR) valuated by spontaneous recording of adverse effects accor...ng to its dosing time. In Figure 1, the number of patients who experienced at least one adverse effect was significantly lower for the evening group (Chi-Square test : p < 0.01) In figure 2, the number of adverse effects was significantly lower for the evening group (Student t test : p < 0.03).

For the overall assessment by patient there was only a trend in favor of the evening group (Chi Square test · p = 0.10, figure 3).
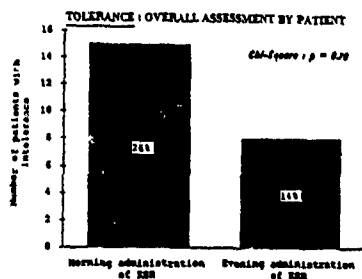
Figure 3 - Tolerance of Ketoprofen sustained-release (KSR) evaluated by overall assessment by patient according to its dosing time. A trend was observed in favor of evening group but the difference was not statistically significant (Chi-Square test : p = 0.10).

. Comparative efficacity of the two groups ·

No statistically significant difference was observed for visual pain-scale (Table II) and overall assessment by physician and patient figures 4 and 5). However for verbal pain-scale, there was a trend in favor of evening group (Chi-Square test : p = 0.10).

Table II - Comparative efficacy of the two groups evaluated by visual pain scale measured at end of trial.

	Morning administration N = 57	Evening administration N = 57	Student t test
Visual pain scale (cms)	38,9 ± 3,3	34,8 ± 3,4	N.S.
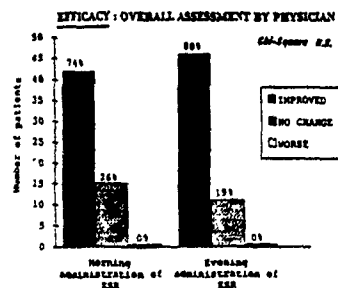
Figure 4
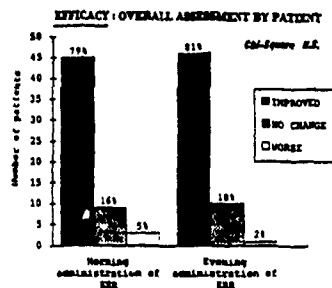
Figure 5

Figure 4 and 5 - Efficacy of Ketoprofen sustained-release (KSR) evaluated by overall assessment by physician (Figure 4) and patient (Figure 5). No statistically significant difference was observed (Chi-Square test N.S.)

. Influence of circadian variation of pain intensity :

In this study, this parameter did not seem to interfere with the results. It could be due to the small sample size of this trial.

DISCUSSION

This study clearly found a better tolerance for the evening administration of KSR in comparison with the morning one. For efficacy no statistically significant difference was observed between the two groups of patients. These results are consistent with those of an other chronotherapeutic trial performed with an other antiinflammatory agent (Indometacin) in osteoarthritis (4). Such data could be explain by a higher peak in the morning for ketoprofen plasma levels which might increase adverse effects (1). A lower gastric mucosal resistance in the morning could be an other mechanism (5).

CONCLUSION

The evening administration should be preferred for ketoprofen sustained-release used in the treatment of osteoarthritis. Similar chronotherapeutic trials must be performed with other antiinflammatory agents before extrapolating such results to all these drugs.

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TENOXICAM CHRONOTHERAPY OF RHEUMATIC DISEASES

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ABSTRACT

A double-blind, placebo-controlled, randomized and cross-over
chronotherapeutic study of piroxicam (P: 20 mg/24 hr) and tenoxicam
(T : 20 mg/24 hr) was performed in 26 ambulatory patients. The aim
of the present study was to evaluate both effectiveness and
tolerance of T at 3 different Rx times : 08 h (8 a.m.), 12 h (noon)
and 20 h (8 p.m.) each one tested during a 14 day span.

1. The tolerance of either T or P was excellent according to both
patient and rheumatologist reports.
2. A circadian rhythm of symptoms persisted whatever the Rx time.
3. Rx time of T at 08 h or noon appeared to be optimal in most
cases.
4. The beneficial effect of T at the optimal once-a-day Rx time was
observed over the 24 h span.
5. In OAH patients, control of stiffness was greater $(P < 0.005; \chi^2 = 7.1)$ than that of pain at the optimal Rx time of T.

KEYWORDS

Rheumatoidal diseases. Tenoxicam. Chronotherapy

BACKGROUND

The NSAID tenoxicam (T) has a rather long half-life (3 days) with
a plasma steady state of 10-15 g/ml reached after a one week span.
The protein binding is 99.2 %. Two questions were raised : is the
effectiveness of T dependent upon the dosing time (Rx time) ? If
so, is there an optimal Rx time of T with regard to, for example,
the treated disease ?

AIM OF THE STUDY

The aim of the present study was to evaluate both effectiveness and
tolerance of T at 3 different Rx times: 08 h (8 a.m.), 12 h (noon)
and 20 h (8 p.m.), each tested during a 14 day span.

PATIENTS

26 ambulatory patients volunteered for the study
- 11 with an ankylosing spondylarthritis (AS)
  (8 males : 22-68 yr ; 3 females : 23-47 yr);
- 8 with a rheumatoid arthritis (RA)
  (8 females : 22-68 yr) ;
- 7 with osteoarthritis of the hip (OAH)
  (2 males : 63-71 yr ; 5 females : 44-65 yr).
Patients were synchronized with a diurnal activity from 08 to 24 h
and a nocturnal rest. Criteria of inclusion took into account a
precise diagnosis of disease, its relative stability over 2 months,
no change (both dose and Rx time) in associated medications (e.g. D-
penicillamine in RA) but no past or present use of corticoid or
other NSAIDs.

METHODS

A double-blind, placebo-controlled, randomized and cross-over
chronotherapeutic study of piroxicam (P : 20 mg/24 hr) and
tenoxicam (T : 20 mg/24 hr) was performed during 4 spans of time.
(Figure 1).
Each patient was given 3 capsules a day, with T at the selected Rx
time and placebo for the others (double blind). A 14 day pre-trial
span, with P at 08 h and placebo at 12 h and 20 h) was programmed
to gather reference values and to check each patient's compliance
and adequate understanding of tests to be performed. Then, each
patient experienced the other 3 spans in randomized order.
During the second half (7 days) of the 4 spans, each patient was
asked to self-rate at fixed hours (08 h, 12 h, 16 h, 20 h and 00 h)
both pain and stiffness using a visual analogue scale with
arbitrary units : a.u. (Huskinson test).
Time series were analyzed both indivudally and as sub-groups using
ANOVA, cosinor, χ 2 test, etc. as statistical methods.
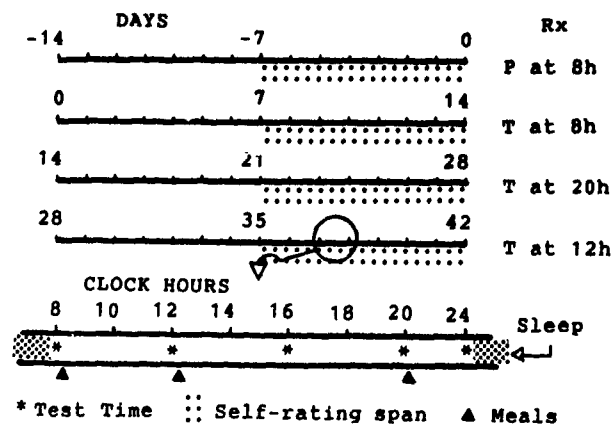
Figure 1

RESULTS

ANKYLOSING SPONDYLARTHRITIS

Since no sex-related differences were validated (cosinor and ANOVA)
data of all subjects with AS were pooled for further time series
analyses. Chronograms show that even under treatments, a
statistically significant circadian rhythm was detected in both

osteoarthritis of hip
pain

Figure 4
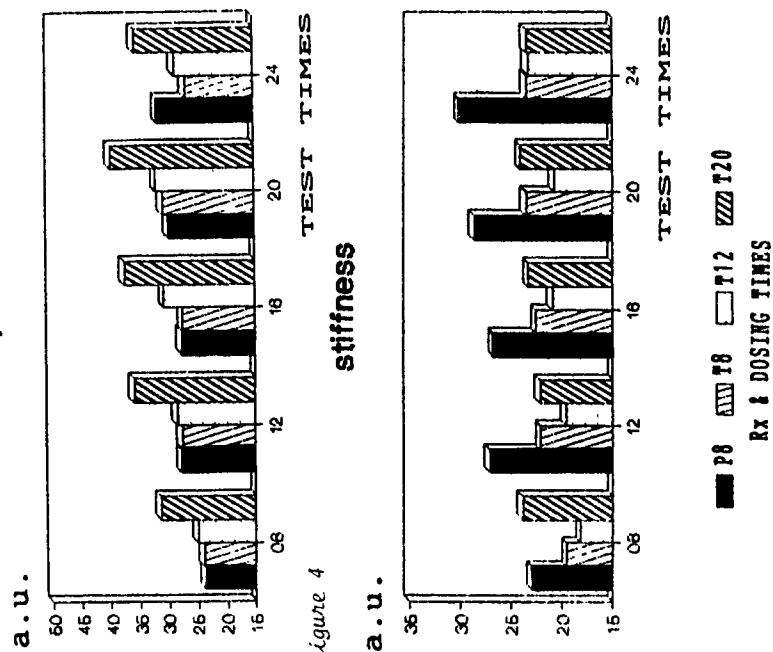
stiffness

Figure 5

ankylosing spondylarthritis
pain
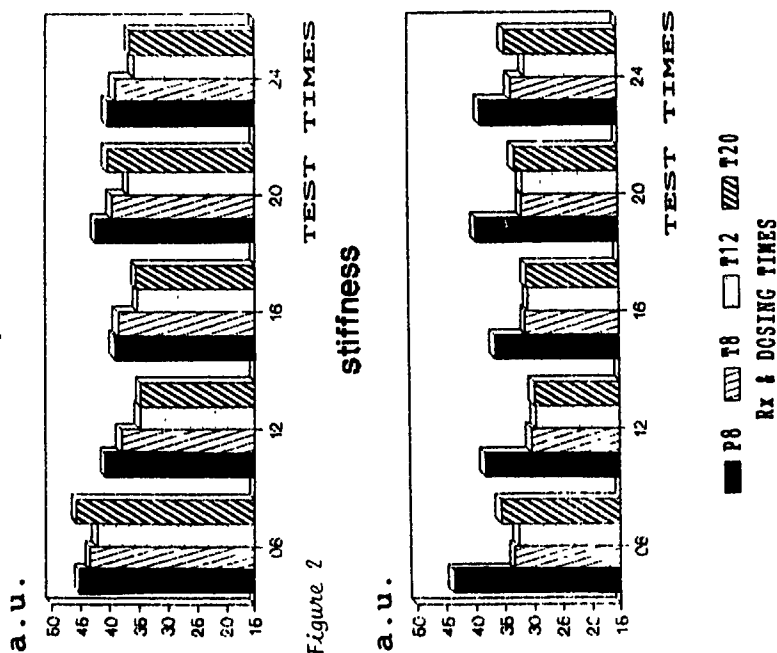
Figure 2

stiffness

Figure 3

pain (figure 2) (P < 0.03) and stiffness (figure 3) (P < 10^{-4}). Peak time occurred in morning hours (around 08 h) as a group phenomenon (Cf. Table 1). A dosing time-dependent change in the effectiveness of T was also validated for both pain (P<0.0004) and stiffness (P< 10^{-4}) (figures 2, 3), with Rx at 12 h (T12) being the most effective. Moreover the latter effect was observed at all test times which means that the beneficial effect of Rx at noon persisted during the 24 hr span.
Data obtained from individual time series (cosinors) were χ^2-tested. This method also shows that T12 was the best dosing time (P < 0.05 for both pain and stiffness).

RHEUMATOID ARTHRITIS

A statistically significant circadian rhythm, with a morning peak at 08 h was validated (cosinor and ANOVA) for both pain (P<0.03) and stiffness (P < 0.002) despite treatments with oxicams.
A dosing time-dependent change of T was validated for both pain and stiffness (P < 10^{-4}). The best efficiency corresponded to either T8 or T12 in comparison with T20, particularely in the control of pain. The beneficial effect of Rx at 08 h or 12 h persisted during the 24 hr span. These results were confirmed with a χtest performed from data of individual cosinor (Table 1) (pain with P < 0.0025 and stiffness with P < 0.05).

OSTEOARTHRITIS OF HIP

A statistically significant circadian rhythm with an evening peak at 20 h was validated (cosinor and ANOVA) for both pain (P< 0.0005) (figure 4) and stiffness (P<0.03) (figure 5) despite treatment with oxicams. Individual analyses confirmed this fact (Table 1).
A dosing-time-dependent change of T was validated for both pain (P< 0.0005) and stiffness (P < 0.0001). With regard to pain, T8 and T12 were more effective than T20 while with regard to stiffness, T12 was a more effective dosing time than T8 and T20. These results were also confirmed with a χ^2 test performed from data of individual cosinors (pain with P < 0.05 and stiffness with P<0.025). Moreover, a better control of stiffness than pain was also validated in OAH (P < 0.0005; χ^2 = 7.1). This was not the case for the two other rhumatoic diseases.

PEAK TIME OF SYMPTOMS AND OPTIMAL DOSING TIME OF TENOXICAM

Disease (No of patients)	PAIN		STIFFNESS	
	Peak time	Best Rx time	Peak time	Best Rx time
AS (11)	Morning (6/11) Evening (5/11)	12 & 8 h (8/11)	Morning (6/11) Evening (5/11)	12 & 8 h (8/11)
RA (8)	Morning (8/8)	8 & 12 h	Morning (8/8)	8 & 12 h (6/8)
OAH (7)	Evening (6/7)	8 & 12 h (6/7)	Evening (7/7)	12 (7/7)

Differences were validated with ANOVA and χ^2 test.

TOLERANCE

In this study, neither T nor P treatments were associated with the occurrence of non-desired effects, whatever the dosing-time, according to both patient and rheumatologist reports.

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CHRONOTHERAPY OF PRIMARY OPEN-ANGLE GLAUCOMA

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ABSTRACT

In 12 cases with 15 eyes of primary open−angle glaucoma that received 0.5% timolol or combined 2% pilocarpine twice to four times a day, an eye drop time schedule based on the measurements of circadian rhythms of intraocular pressure (IOP) could control the IOP under 20 mmHg more efficiently than the routine schedule, and made the circadian variation less and prevented from further visual damages during follow up of 5 to 13 months.

KEYWORDS

Open−angle glaucoma; timolol; pilocarpine; chronotherapy.

INTRODUCTION

In diagnosis and treatment of glaucoma, valuable additional information can be gained by circadian IOP monitoring (Drance, 1960). It has been proposed that in eyes that have already sustained glaucomatous damage, the progression may be explained by the presence of previously undocumented IOP peaks occurred at times outside of normal office hours (Wilensky et al.,1987). Routine timolol/pilocarpine combination eye drops in open−angle glaucoma caused a statistically significantly greater reduction of the IOP than that achieved by timolol alone (H:vding and Aasved, 1987), but we found in clinic that the peaks of IOP circadian rhythm often could not be controlled within the normal range with them, yet. This study was an attempt to control IOP within the normal range throughout the day and night only by changes of eye drop time of the same drugs based on preceded measurements of the circadian rhythm of IOP.

MATERIALS AND METHODS

12 patients (2 females and 10 males, aged 28−50 years) with 15 eyes of primary open−angle glaucoma were studied. They had typical glaucomatous manifestation, including permanent increase of IOP, decrease of visual acuity, visual field loss and enlargement of the optic cup to disc ratios, and were admitted 3 to 5 days after stopping previous glaucoma medication., Each patient was presumably diurnally active, and had consecutive 48 hr measurements of circadian IOP every three hrs using a Schiotz tonometer before the study began. As the self−control, they first received a routine schedule of timolol 0.5% eye drops twice daily (07:00 and 16:00) for 3 to 5 days. For 10 eyes among them, which IOP at the office time (07:00−18:00) could not controlled by timolol alone, combined pilocarpine 2% eye drops twice to four times a day (07:00, 11:00, 14:00, 18:00) were added. After the routine schedule, the consecutive 48 hr measurements

of circadian IOP were repeated and compared to that before the treatments. Owing to that the peak of circadian IOP rhythms could not be controlled within normal range, then the routine schedule was changed to a chronotherapeutic schedule based on the measurements of circadian rhythms of IOP: drop time at 11:00 and 22:00 while the peaks at 02:00—05:00, drop time at 11:00 and 19:00 while the peaks at 20:00—22:00. For the patients that need four time drops a day, other twice eye drops were added at equal interval among them. The circadian IOP for consecutive 48 hrs were measured again after 3 to 5 days. Follow up of the circadian IOP and the visual function measurements was done after 5 to 13 months keeping on the chronotherapeutic schedule.

RESULTS

The results showed that there were significant circadian rhythms of IOP in the eyes with primary open—angle glaucoma, and the peaks in the early morning before the study. Even though the routine administration of timolol and/or pilocarpine could decrease the IOP significantly, the peaks of circadian IOP at 02:00—05:00 was still over 20 mmHg and the circadian amplitudes seemed to increase, whereas the chronotherapeutic schedule controlled more efficiently the peaks under 20 mmHg and made the circadian variation less (Fig.1A,B). The Cosinor analysis of the results was shown in Table 1. Besides, follow up of these patients received the chronotherapeutic schedule for 5 to 13 months indicated no a case had further decrease of the visual acuity and the visual fields, and enlargement of the optic cup to disc ratios. The circadian IOP rhythms after keeping on the chronotherapeutic schedule for a long term (Fig.2) were similar to the short—term results after the schedule began.
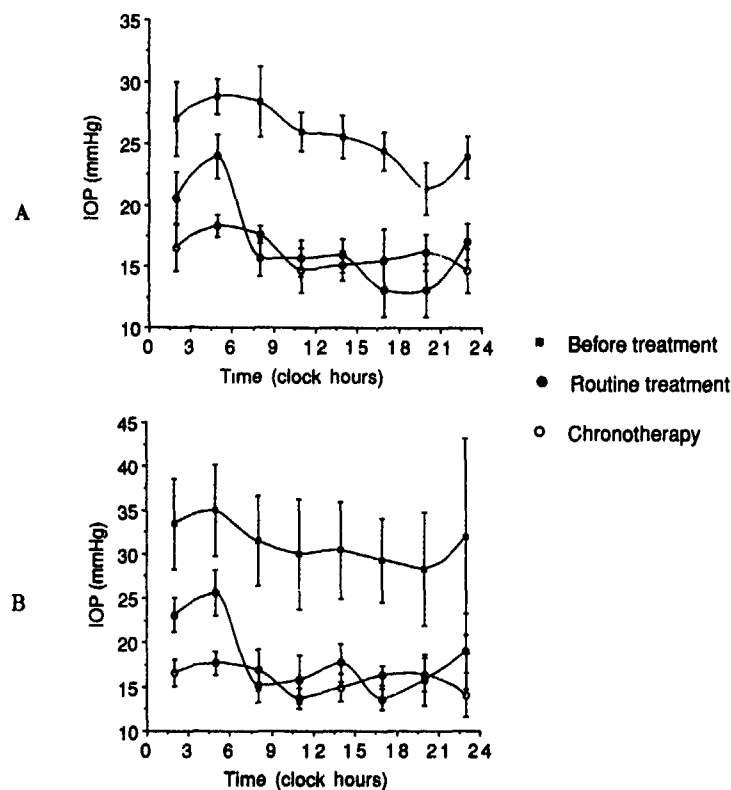
Fig. 1. Mean chronogram of circadian IOP in 12 patients with 15 eyes of primary open—angle glaucoma. A: 5 eyes received only timolol treatments. B: 10 eyes received both timolol and pilocarpine.
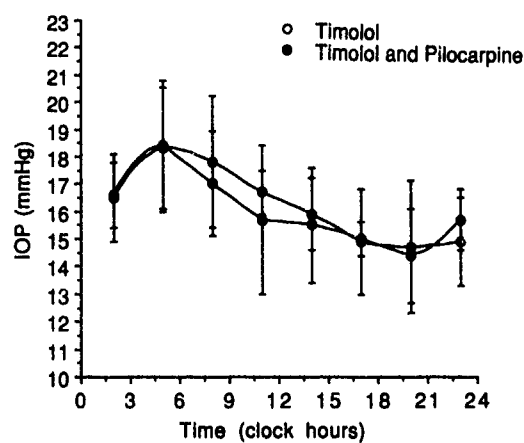
Fig. 2. Mean chronogram of the circadian IOP from follow up after 5 to 13 month.

Table 1. Cosinor analysis of circadian IOP of 15 eyes with open
angle glaucoma   before the study and 3 to 5 days after
two kinds of eye drop schedules.

Drug	No	Mesor (95% CL[#])	Amplitude (95% CL)	Phase Angle (95% CL)	P Value[+]
Timolol	1	25.7 (25.0−26.4)	3.0 (2.0−3.9)	−104 (−123− −86)	<0.0001
	2	16.9 (16.1−17.8)	4.1 (2.9−5.3)	−64 (−81− −47)	<0.0001
	3	16.1 (15.5−16.6)	1.2 (0.4−2.0)	−75 (−115− −35)	0.02
Timolol and Pilocarpine	1	31.2 (29.8−32.6)	2.5 (0.5−4.5)	−64 (−109− −19)	0.04
	2	18.2 (17.4−19.0)	4.2 (3.1−5.3)	−49 (−64− −34)	<0.0001
	3	15.8 (15.3−16.3)	0.8 (0.1−1.5)	−50 (−97− −3)	0.06

+ Analysis of variance for testing zero amplitude.
# 95% Confidence limit.
No. 1, 2, 3 mean before the study and 3 to 5 days after the routine or chrono.herapeutic
schedules, respectively.

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DISCUSSION

The acrophases of circadian IOP of the eyes with primary open—angle glaucoma were similar to that in normal eyes reported previously (Cui et al., 1988), though the peak values were much higher than that in normal eyes. It is relevant to prevent further glaucomatous visual damage to control the IOP throughout the day and night within normal range. In this study, the routine eye drop time using glaucoma medication might miss the peak time (e.g., 02:00—05:00) of circadian IOP rhythm, so could not cut the peak down efficiently, while the chronotherapeutic schedule made eye drop time near or before the peak time of circadian IOP rhythms as possible, and ensure the peaks of circadian IOP fall into the save range. It suggests that only change of administration time based the circadian rhythms of IOP, even not change the dose can improve short and even long term efficacy of timolol or/and pilocarpine on primary open—angle glaucoma.

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THE EFFECT OF CHRONOTHERAPY WITH A 24-HOUR SUSTAINED RELEASE THEOPHYLLINE PREPARATION (UNIPHYL) ON A.M. AND P.M. DIFFERENCES IN AIRWAY PATENCY AND RESPONSIVENESS

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BACKGROUND

Airway tone expresses a circadian rhythm (Reinberg et al. 1972) (Smolensky et al. 1981). Expiratory flow rates of normal subjects have their peak in the late afternoon and their trough in the early morning hours (Smolensky et al. 1981) (Hetzel 1981) In patients with asthma the same rhythm is present, but its amplitude is exaggerated (Smolensky et al. 1981) (Hetzel 1981). This may be the result of an increase in the inflammation of the airways during the night secondary to circadian dips in the levels of endogenous cortisol and epinephrine (Barnes 1988). The ensuing bronchial hyperresponsiveness probably contributes significantly to the pathogenesis of nocturnal asthma (Barnes 1988). This can be shown in the pulmonary function laboratory by an increased sensitivity to histamine during the night (Ryan et al. 1982). It manifests itself clinically as nocturnal wheezing, cough and awakening commonly experienced by patients with asthma (Turner-Warwick 1984).

An important clinical objective in treating these patients is to reduce their nocturnal rise in bronchial hyperresponsiveness. Sustained release theophylline administered at night is known to be effective in the treatment of nocturnal asthma (Barnes et al. 1982) (Neuenkirchen et al. 1985) (Arkinstall 1988). The success of this rhythm-adapted dosing strategy is due in part to better temporal matching of high serum theophylline concentrations with maximum bronchoconstriction (Smolensky et al. 1988). Moreover, high theophylline concentrations can be maintained for a prolonged period of time by taking advantage of the chronokinetics of some preparations which exhibit a slower absorption during the night without loss of bioavailability (Scott et al. 1981) (Busse et al. 1985). Although theophylline has been a very important agent in the therapy of asthma for many years, its role in the treatment of bronchial hyperresponsiveness is controversial, and therapeutic strategies for its use are based predominantly on its bronchodilating properties (Hendeles et al. 1983) (Mitenko et al. 1973) (Fairshter et al. 1986). Several studies have documented the ability of theophylline to reduce the bronchoconstrictive effects of histamine (Cartier et al. 1986) (Crimi et al. 1987), histamine and methacholine

(McWilliams et al. 1984) (Magnussen et al. 1987) and antigen (Falliers et al. 1976), especially during the late phase reaction (Pauwels et al. 1985). These studies, however, are contradicted by other work (Dutoit et al. 1987) (Cockcroft et al. 1989).

In the present study we present evidence that theophylline alters the circadian variation in response to histamine of patients with asthma, and that the effect of theophylline administration is greater when it is administered in the evening.

METHODS

Patients with asthma of moderate severity, defined by an FEV_1 of 50-80% of predicted and 15% reversibility following an inhalation of a ß-agonist, were eligible for the study. The patients were non-smokers, aged between 18 and 65 years. All patients had received stable oral theophylline therapy for at least one month prior to enrollment in the study. All showed airway responsiveness as determined by histamine challenge (PD_{20}) (Cockcroft 1985) which was at least twice greater in the morning than in the evening. The use of ß-agonists was permitted. Therapy with corticosteroids, cromolyn, macrolide antibiotics, and/or cimetidine was not allowed Among exclusion criteria were pregnancy, recent respiratory infection, recent influenza vaccination, participation in night shift work, and variable sleep schedule.

The dose of Uniphyl required to achieve a serum theophylline concentration of 12-20 µg/ml 12 hours after dosing was established for each patient. This dose was administered throughout the study. The patients were randomly assigned to an AM or PM treatment regimen. After 2 weeks of this treatment they were crossed over to the opposite regimen. The patients underwent histamine challenges (Cockcroft 1985) at 7 AM and 5 PM within the same 24-hour period. The sequence of these challenges was assigned by a randomization schedule. Challenges were performed following washout from theophylline at the start and again at the conclusion of the study, and on days 7 and 14 of both treatment regimens. Theophylline concentrations were measured at the time of each challenge. PD_{20} values were calculated by a logarithmic interpolation between the last 2 data points.

A repeated measures analysis of variance was used to determine the significance of AM/PM differences in theophylline concentrations, FEV_1 and PD_{20}. Tests for carry-over effect were carried out according to Jones and Kenward (1989).

RESULTS

Thirteen patients were enrolled and 11 patients completed the study. No carry-over effect between study groups was demonstrated. Patients in both study groups achieved peak theophylline levels 12 hours after dosing with Uniphyl. There was no significant change in FEV_1 values. There was a reduction in the AM/PM difference in PD_{20} in both study groups. This reached a high level of significance for the PM group ($p < 0.05$) (see Figure 1). Moreover, there was a significant difference between the AM and PM study groups ($p < 0.05$) (see Figure 2). A significant relationship was demonstrated between serum theophylline concentrations and airway reactivity measured in the AM for the patients receiving theophylline in the evening ($p < 0.0005$).

Morning - Evening Changes in PD20
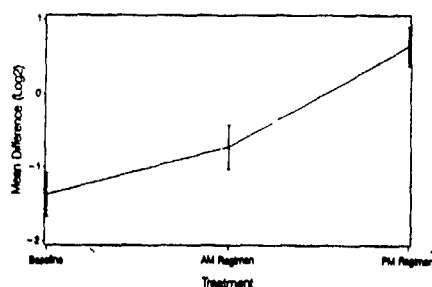
Fig. 1
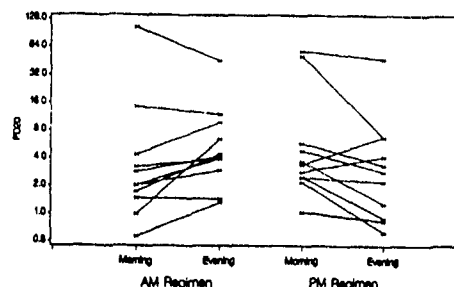
Fig. 2

CONCLUSIONS

Our results show that the circadian pattern of airway reactivity is affected by the time of administration of theophylline. Evening dosing with theophylline eliminates the AM peak in airway hyperresponsiveness, and a significant relationship exists between theophylline concentrations and airway responsiveness in this group of patients. Interestingly, these changes in airway reactivity are not reflected by changes in FEV_1.

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DIURNAL VARIATION IN PEAK FLOW RATE IN CHILDREN WITH MILD ASTHMA. EFFECTS OF TREATMENT WITH BUDESONIDE AND TERBUTALINE

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ABSTRACT

In a double blind, randomized placebo controlled study of 8 weeks
we compared the effects of treatment with either budesonide (200µg
bid) and terbutaline (500µg qid) or terbutaline alone on bronchial
hyperresponsiveness, peak flow recordings and the amplitude of
diurnal variation in peak flow rate (PEFR) in 27 children with
mild asthma. Fifteen children were treated with terbutaline and
placebo (group T) and 12 children with the combination of budeson-
ide and terbutaline (group bT). PC_{20}-histamine increased sig-
nificantly in group BT, and was unchanged in group T. Morning PEFR
(8 am) improved significantly in both groups ($p < 0.05$). Afternoon
PEFR (4 pm) only improved significantly in group BT ($p < 0.05$).
After 7 and 8 weeks of treatment nocturnal PEFR (4 am) was sign-
ificantly higher in group BT compared with group T ($p < 0.05$). The
amplitude of diurnal variation in PEFR over the day was calculated
as the difference of the lowest and highest PEFR expressed as a
percentage of each days mean PEFR. The amplitude of diurnal
variation in PEFR including post-bronchodilator values, decreased
from 17.5% to 15.4% in group T (ns) and from 15.8 to 10.5% in
group BT ($p < 0.05$). We conclude that even in children with a mild
bronchial obstruction additional treatment with budesonide on
addition to treatment with inhaled terbutaline leads to an im-
provement of afternoon PEFR, nocturnal PEFR and a decrease in
amplitude of diurnal variation in PEFR when compared with treat-
ment with terbutaline alone.

KEY WORDS

Asthma, diurnal variation, PEFR, budesonide, terbutaline, bronchi-
al hyperresponsiveness

INTRODUCTION

One of the features of asthma is an increased circadian rhythm in airway calibre with peak and trough values at 4 pm and 4 am, respectively (Hetzel and Clark, 1980). The exact underlying mechanism is still uncertain. Recent studies have found extensive epithelial destruction in the airways of even mild asthmatic patients (Beasley et al, 1989). It has been suggested that these inflammatory processes may play an important role in bronchial hyperresponsiveness and symptoms of asthma. Kraan et al. (1985) demonstrated in adults with asthma that treatment with inhaled corticosteroid induced a decrease in bronchial hyperresponsiveness and diurnal variation.

The aim of the present study was to investigate the effects of treatment with inhaled corticosteroids on addition to inhaled terbutaline on bronchial hyperresponsiveness and the amplitude of diurnal variation in PEFR in mild asthmatic children.

PATIENTS AND METHODS

Twenty-seven asthmatic children aged 7-14 years (mean 10.3) were selected from our outpatient department. All children had a history of episodic shortness of breath and/or wheeze and a measurable bronchial hyperresponsiveness to histamine, defined by a PC_{20}-histamine below 8 mg/ml. Baseline FEV_1, had to be above 70% of the predicted value. None of the children was treated with inhaled corticosteroids the preceding 6 months or used oral corticosteroids regularly. Children with concomitant disease or a recent respiratory tract infection were excluded. Before entering the study the children discontinued their maintenance treatment and used only a beta-2-agonist on an "if needed" basis.

STUDY DESIGN

The children were randomly assigned to one of the two treatment groups. After a single blind run-in period of two weeks, receiving terbutaline (500µg, qid) and placebo, the children were treated with either budesonide (200 µg bid) and terbutaline (500µg qid) or terbutaline and placebo. PC_{20}-histamine was measured before and after the run-in period and after 4 and 8 weeks of double blind treatment. At home, morning (8 am) and afternoon PEFR (4 pm), before and 10 minutes after inhalation oi terbutaline (mini-Wright, best of three attempts), were recorded in the run-in period, week 3 + 4 and week 7 + 8 of double blind treatment. Nocturnal PEFR (4 am) was determined twice in the week prior to each visit at the clinic. The amplitude of the diurnal variation in PEFR over the day was calculated as the difference of the lowest and highest PEFR, expressed as a percentage of each days mean PEFR. Biweekly mean peakflow recordings measured in week 3 + 4 and week 7 + 8 were compared with the mean values in the run-in period. Individual changes and comparison of PC_{20}-histamine and peakflow values in both groups were analyzed with the use of the Student's t-test for paired and unpaired observations. Data are expressed as mean and standard error of the mean (SEM).

RESULTS

Twenty-seven children completed the study. Twelve children were
treated with budesonide and terbutaline (group BT) and 15 children
with placebo and terbutaline (group T). The characteristics of the
patients are illustrated in table 1.

Table 1: Clinical data of the patients at visit 1
(mean ± s. e. m)

	BT	T
Boys/girls	7/5	13/2
Age (years)	11. 4 ± 0. 7	9. 8 ± 0. 5
FEV$_1$ % pred	93. 3 ± 3. 4	92. 5 ± 2. 9
VC % pred	98. 0 ± 4. 4	94. 0 ± 2. 8
PC$_{20}$ histamine (mg/ml)	0. 51	0. 89

At the entry into the study both groups were comparable with
respect to sex, age, baseline pulmonary function and PC_{20}-his-
tamine. Mean PEFR values are shown in table 2.

Table 2: Biweekly mean PEFR values

morning	BT: a	328. 6 ± 26. 2	351. 3 ± 30. 0*	359. 2 ± 26. 6*
	b	356. 5 ± 27. 3	368. 2 ± 28. 3	373. 7 ± 28. 3*
	T: a	325. 1 ± 20. 4	329. 0 ± 21. 9	338. 7 ± 18. 7**
	b	358. 7 ± 22. 1	359. 8 ± 22. 2	369. 4 ± 21. 1**
afternoon	BT: a	359. 2 ± 27. 8	374. 3 ± 29. 1*	382. 1 ± 29. 3***
	b	369. 6 ± 28. 1	385. 4 ± 29. 0**	390. 6 ± 29. 8***
	T: a	353. 4 ± 24. 1	350. 0 ± 22. 5	357. 5 ± 21. 4**
	b	373. 0 ± 23. 5	371. 8 ± 23. 0	380. 3 ± 22. 8**
nocturnal	BT	328. 2 ± 26. 1	354. 4 ± 30. 3	355. 2 ± 13. 3*
	T	310. 7 ± 21. 8	302. 9 ± 26. 9	299. 2 ± 23. 3

before (a) and 10 minutes after (b) bronchodilation
BT = budesonide plus terbutaline; T = terbutaline plus placebo
* p < 0.05 and ** = p < 0.01 : versus run-in period
= p < 0. 05: change from run-in group BT versus change from
run-in group T

After a treatment period of 8 weeks mean morning and afternoon
PEFR, before and after bronchodilation, increased significantly in
group BT. Nocturnal PEFR increased although the increase was not
significant. The amplitude of diurnal variation in PEFR including
post-bronchodilator values decreased from 15. 8 to 10. 5% (p< 0. 05).
When only pre-bronchodilator values were used, the diurnal varia-
tion decreased from 11. 7 to 8. 1%. (ns). The geometric mean PC_{20}-
histamine increased from 0. 30 mg/ml after the run-in period to
0. 68 mg/ml at the end of the trial (p < 0. 05).

After 8 weeks of treatment with terbutaline alone, mean morning
pre-and post bronchodilator PEFR increased significantly. Mean
afternoon pre-bronchodilator PEFR remained stable, but post-
bronchodilator PEFR increased slightly. Nocturnal PEFR decreased
(ns). The amplitude of diurnal variation in PEFR including post-
bronchodilator values decreased from 17.5 to 15.4% (ns) and
decreased from 11.6 to 9.0% when only pre-bronchodilator values
were used (p< 0.01). PC_{20}-histamine changed slightly from 0.82 to
0.75 mg/ml.

DISCUSSION

The results of the present study demonstrate that in children with
mild asthma treatment with the combination of a low dose budeso-
nide on addition to terbutaline leads to a significant improvement
of PEFR recordings and a decrease in amplitude of diurnal varia-
tion in PEFR (post bronchodilator values included) in comparison
with treatment with terbutaline as a single treatment. It has been
demonstrated that bronchial hyperresponsiveness increases during
the night (de Vries et al, 1962), but that an increased bronchial
hyperresponsiveness during the night is not in itself responsible
for nocturnal asthma (van Aalderen et al., 1989). During allergic
inflammatory processes mediators are released leading to an
imbalance of the autonomic nervous system and a disruption of the
airway epithelium (Hogg, 1981). Both factors increase probably
bronchial hyperresponsiveness and diurnal variation in PEFR.
Maintenance treatment with inhaled corticosteroids will lead to
dampening of these inflammatory processes.
The results of the present study indicate that even in mild
asthmatic patients inhaled corticosteroids are of benefit to
diurnal variation in lung function and bronchial hyperresponsive-
ness. This effect is probably due to a reduction of inflammatory
processes in the airways.

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ADRENERGIC RESPONSE IN CHILDREN WITH AND WITHOUT NOCTURNAL ASTHMA ON EXOGENOUS STIMULI

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ABSTRACT

Exogenous stimuli may increase nocturnal airflow obstruction in asthmatic patients. We hypothesized that asthmatic children with nocturnal airflow obstruction may suffer more frequently from a late obstructive reaction (LOR) due to exogenous stimuli during the day than children without nocturnal airflow obstruction. Therefore we investigated the bronchial obstructive reaction pattern after house dust mite (HDM) inhalation and after exercise in a group of asthmatic children with (group I) and without (group II) increased airflow obstruction overnight. Urinary adrenaline and N^τ-methylhistamine excretion was measured during and after both stimuli. The results of both asthmatic groups were compared to the results of age-matched controls. In both groups of asthmatic children we observed a simalar bronchial obstructive pattern after both stimuli. N^τ-methylhistamine excretion increased during the early obstructive reaction (EOR), but not during the LOR after HDM. An impaired adrenergic response in both asthmatic groups was observed, in group I more than in group II, suggesting that the degree of impairement of the adrenergic response is related to the severity of the asthmatic disease state.

Key words: nocturnal asthma, house dust mite, exercise.

INTRODUCTION

Nocturnal airflow obstruction in asthmatic children may be induced by exogenous stimuli. Inhalation tests with allergens have been shown to produce recurrent asthmatic attacks for several nights. Challenges with allergens or exercise have also been found to produce EOR as well as LOR, which may be responsible for the increase in airflow obstruction overnight. We hypothesized that asthmatic children with increased nocturnal airflow obstruction may suffer more frequently from LOR due to exogenous stimuli than asthmatic children without nocturnal airflow obstruction. Therefore

we investigated the bronchial obstructive reaction patterns after
HDM challenge and after exercise in a group of asthmatic children
with   (group   I)   and   without   (group   II)   nocturnal   airflow
obstruction. Additionally we investigated whether any differences
between the two groups were caused by differences in adrenaline
response, or by differences in N^τ-methylhistamine excretion during
and after both stimuli. The results of the two patient groups were
compared to the results of an age-matched group of controls.

METHODS

Selection of the patient groups and their clinical parameters were
published elsewere (Van Aalderen et al. 1989). The study was
performed from September up to and including November 1986 and 1987
to avoid seasonal influences. Fixed times for meals and sleeping
were   set   in   hospital.   On   the   first   day   in   hospital FEV_1
measurements were performed every 4 hours during 24 hours. Times of
measurement   were   08.00,   12.00,   16.00,   20.00,   24.00,   04.00,   and
08.00 hours. Urine samples in 4 hour portions were collected for
the determination of adrenaline and N^τ-methylhistmaine. The values
from the samples from 08.00-20.00 hours served as control values to
the values obtained during both provocation days.

 The HDM provocation tests were performed at 08.00 hours on the
fifth day of admission. FEV_1 values were than measured with
interfalls of 2 hours up to and including 20.00 hours. With the
same interfalls urine samples for the determination of adrenaline
and N^τ-methylhistamine were collected.

 Exercise tests were carried out six weeks thereafter, in order to
avoid carry-over effects from the HDM challenge. The exercise test
was performed at 08.00 hours on a treadmill and consisted of six
minutes running with speed and slope necessary to achieve a heart
rate of 180 beats per minute or more.

The EOR was defined as a 15% fall or more in FEV_1 from the 08.00
hours prechallenge value and the LOR was defined as a 15% fall or
more, occurring 3-10 hours after the EOR, expressed as percentage
difference between the FEV_1 value at a given point in time on the
provocation day and the same time of the day on the control day.

RESULTS

Lungfunction

The obstructive reaction pattern from the two asthmatic groups is shown in table 1. No signficant difference between the two groups in EOR as well as in LOR were observed. No LOR's after exercise were observed.

Table 1. House dust mite challenge

EOR	n	% fall	LOR	n	% fall
Group I	9	37.4±5.7%		7	37.1±6.2%
Group II	9	25.2±3.9%		8	36.4±3.9%

Exercise					
EOR	n	% fall	LOR	n	% fall
Group I	6	28.5±5.4%		0	
Group II	5	24.0±1.8%		0	

Urinary Nτ-methylhistamine excretion

Although a mean increase in Nτ-methylhistamine excretion was observed during the first 2 hours after HDM challenge, this increase did just not reach significance in either group. During the LOR, no increase in Nτ-methylhistamine excretion was observed at all. No increase in Nτ-methylhistamine was observed after exercise during the following twelve hours.

Urinary adrenaline excretion

The adrenaline excretion values in the first two hours after HDM challenge (striped bars) showed no change compared with the values of the control day (open bars) in either patient group in spite of severe airflow obstruction in some patients (fig. 1). After exercise, in group I (left panel, black bar), no increase and in group II (middle panel, black bar) a non significant increase in adrenaline excretion was observed

during the first two hours. The control group (right panel) showed a significant increase ($p < 0.01$) compared to control day values.
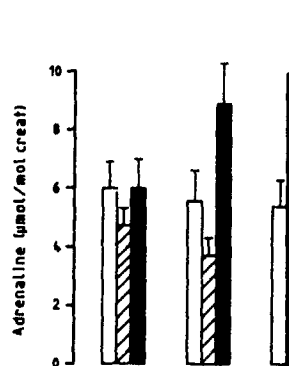
DISCUSSION

This study shows that both groups of asthmatic children had comparable bronchial obstructive reaction patterns on both exogenous stimuli. In contrast to the observations of others we did not find LOR's after exercise. The difference between the study of Lee et al. (1983) and our investigations may be that the children in our study returned to FEV_1 baseline values after the EOR while this was not the case in their study.

In both patient groups, urinary N^τ-methylhistamine excretion during the first two hours after HDM challenge showed a tendency to increase compared with the control day. This observation points in the same direction of observatio s of Keyzer et al. (1984) and indicate direct involvement of mast cells. During the LOR no increase in N^τ-methylhistamine was observed.

Despite severe airflow obstruction after HDM challenge during the EOR in both patient groups no increase in adrenaline excretion was observed. There was a striking difference in urinary adrenaline excretion between the three groups after exercise. Group I, the group with a nocturnal decrease in FEV_1 values, and thus more severe asthma, did not show an increase at all, group II showed a non-significant increase, and the healthy children showed a significant increase in adrenaline excretion during the first two hours after exercise despite a comparable exercise load in all three groups. These results suggest an impaired adrenergic response after challenges in asthmatic children which seems to be related to the severity of the disease.

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NOCTURNAL ASTHMA IN CHILDREN; UNDERLYING MECHANISM

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ABSTRACT

We investigated whether an imbalance in the autonomic nervous system, and/or inflammatory mediators like histamine, are responsible for the nocturnal increase in airflow obstruction. Two groups of nine allergic asthmatic children, were selected, one group with (group I), and one group without (group II) increased nocturnal airflow obstruction. The results of the two groups of asthmatic children were compared to the results of an age- matched control group. On the study day, values of forced expiratory volume in one second and electrocardiogram recordings of one minute were obtained every 4 hours during 24 hours. Heart rate and sinus arrhythmia gap were used to express vagal activity indirectly. Urine was collected in periods of 4 hours between the measurements, and used for the determination of adrenaline and N^τ-methylhistamine. In group I, overall N^τ-methylhistamine excretion was on a higher level and significantly higher overnight compared with the values of both other groups. These observations might indicate that nocturnal airflow obstruction is caused by increased release of inflammatory mediators overnight. Endogenous orthosympathetic and parasympathetic stimulation was found to be of minor importance to the nocturnal regulation of the airway diameter.

Key words: nocturnal asthma, histamine, autonomic nervous system.

INTRODUCTION

Nocturnal dyspnea is a common symptom in asthmatic children. Variations in the autonomic nervous system and/or fluctuations in inflammation of the airways may play an important role in the regulation of the airway diameter.

We investigated in two groups of asthmatic children, group I with, and group II without nocturnal airflow obstruction and a group of age-matched controls (C) whether 24-hour variations in autonomic nervous system balance, and in N^τ-methylhistamine as a

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representative of mediator release, contributed of to the increase of airflow obstruction overnight.

METHODS

Selection of the two groups of allergic asthmatic children was based on peak expiratory flow rate (PEFR) values measured every 4 hours during 24 hours on three consecutive days at home. Group I had an amplitude (the difference between the highest and lowest PEFR value measured over 24 hours expressed as percentage of the mean value) of $\geq$ 20% and group II < 15%.

The study was performed in hospital. To avoid seasonal influences the study was performed from september up to and including november 1986 and 1987. Fixed times for meals and sleeping were set in hospital. Three days before and during the study all medications were withdrawn.

On the day of admission, values of forced expiratory volume in one second (FEV_1) and electrocardiogram recordings of one minute were obtained every 4 hours during 24 hours. Heart rate (HR) and sinus arrhythmia gap (SAG) were used to express parasympathetic activity indirectly. Urine was collected in portions of 4 hours in between the measurements, and samples were used for the determination of adrenaline, noradrenaline, and N^τ-methylhistamine.

RESULTS

Lung function

The lung function parameters of the three groups on the day of admission are presented in fig.1.
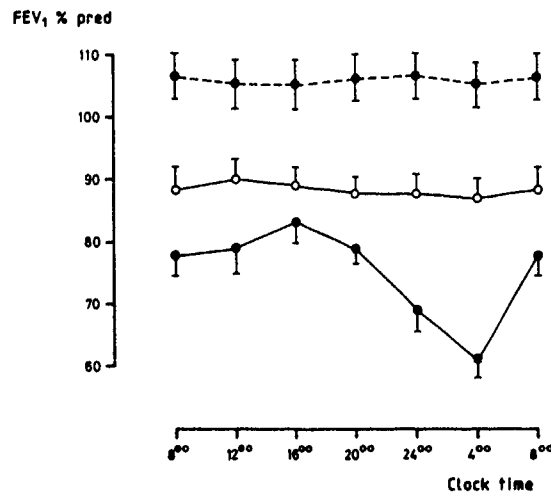
Parasympathetic activity

No increase in parasympathetic parameters, HR and SAG, was observed in all three groups, as is shown in table 1.

Table 1. Parasympathetic parameters of the three groups.

	08.00	12.00	16.00	20.00	24.00	04.00	08.00
Heart rate (beats/min.)							
I :	84.9±4.0	75.8±2.5	82.4±3.7	90.9±6.1	76.2±3.9	68.7±2.4	80.9±3.6
II:	79.3±4.4	71.3±3.4	73.8±3.2	76.0±3.6	68.5±2.5	69.1±4.0	74.6±4.3
C :	83.2±4.7	75.6±2.8	78.3±2.9	81.1±3.6	75.4±3.7	72.7±3.6	82.3±4.7
Sinus arrhytmia gap							
I :	21.3±2.4	20.5±2.0	19.8±1.6	17.0±2.4	16.9±2.4	20.5±2.7	21.3±2.0
II:	23.1±2.9	24.2±3.3	26.8±2.5	23.8±3.0	22.6±1.7	22.1±2.1	23.7±1.9
C :	19.5±1.4	22.7±1.9	20.9±1.9	20.9±1.9	19.8±1.7	19.3±1.3	19.5±1.4

Orthosympathetic activity

No significant differences were observed in urinary adrenaline excretion between the three groups at any point in time. All three groups showed a comparable fall in adrenaline during the night.

Urinary N^τ-methylhistamine.

Group I showed a significant increase in N^τ-methylhistamine excretion overnight. No variation in the other two groups was observed. N^τ-methylhistamine values of group I are overall higher as compared to the ones group II. N^τ-methylhistamine values of group I and of group II are higher than in the control group except for the 04.00-08.00 hours values (fig. 2).
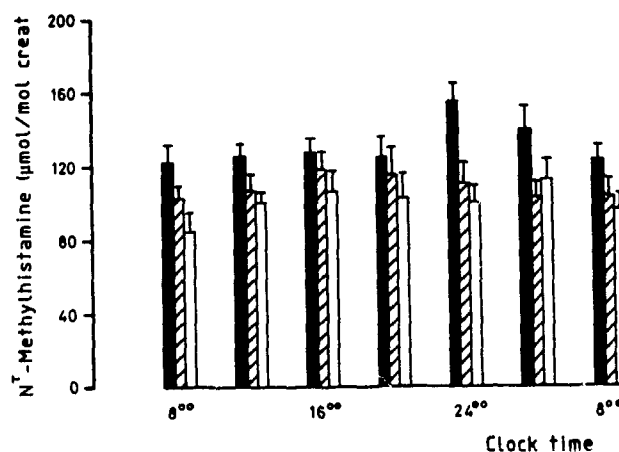
DISCUSSION

During the night excretion levels of N^τ-methylhistamine of the group with nocturnal airflow obstruction were significantly higher compared with ... two other groups. During the day as well as during the night both patient groups showed higher N^τ-methylhistamine excretion levels than the control group, and group I showed higher levels than group II. These increased levels reflect the enhanced degree of inflammation at night. Data of Martin et al. who showed in adult patients an increase in inflammatory cells in the broncho alveolar lavage fluid at night support this hypothesis. Both patient groups were clinically stable during the study. Group I appeared to have more severe asthma since increased airflow obstruction overnight is known to be related to a more severe disease presentation. The difference in excretion levels between the two asthmatic groups indicates that mediator cells of allergic patients with more severe asthma produce a larger amount of histamine than these cells of patients with a less severe state of the disease.

Increased parasympathetic activity does not, in contrast to the observations made in adult patients with asthma, play an important role in nocturnal airflow obstruction in asthmatic children. Furthermore, our data show that endogenous adrenaline production is not stimulated by the fall in FEV_1 values overnight, indicating that adrenal stimulation may be insufficient in this group of patients. The general idea is that an increased nocturnal airflow obstruction is the consequence of a nocturnal fall in circadian adrenaline. We show this not to be the case as all three groups have a comparable decline in adrenaline overnight. Therefore, the observation that group I had an increase in N^τ-methylhistamine cannot be solely due to a fall in adrenaline at night. More basic abnormalities in cellular type and/or activation in the airways must be present at night.

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DAY-NIGHT PATTERN OF ISOPROTERENOL (ISO) USE FOR RELIEF OF ACUTE ASTHMA SYMPTOMS

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For spans of 14 to 28 consecutive days, 11 day-active asthma
patients treated with theophylline (n = 7) at 12-hr intervals and
beta-agonists aerosols (n = 11) via metered dose inhalers (MDI) every
4-6 hrs were followed for reliance upon short acting beta-agonist ISO
aerosol for the relief of breakthrough asthma symptoms, such as tight
chest and wheeze. The ISO aerosol canisters were fitted with chronolog
nebulizers (Forefront Technology; Denver, Colorado, USA), a device
which incorporates a microchip for recording the calendar date and
clock time of use. In addition, patients self-recorded their peak expi-
ratory flow (PEF) every 4 hr during the diurnal activity span and also
upon awakening from sleep at night due to asthma. Daily diaries were
maintained to follow health status and medication use. Over all pa-
tients, there was agreement 88% of the time between data recorded in
the diaries and the record of the chronolog nebulizer. Reliance on ISO
for the immediate relief of acute asthma was not random during the
24 hr. Nearly 70% of ISO use occurred between 2000 and 0800; 22%
occurred between 0700-0800 and 2300-0000. ISO use was greatest
between 0400-0800 and least between 1600 and 2000 with the difference
in use being statistically significant (paired t-test; p = 0.034). Fur-
ther analysis of the data revealed ISO use not to be correlated with the
24-hr mean PEF (r = 0.26; p > .05). However, ISO use was correlated
in a statistically significant manner with the circadian amplitude of the
rhythm in PEF (r = 0.60; P < 0.025). The nocturnal excess in the
reliance upon ISO for relief of acute asthma symptoms while patients
were adhering to a homeostatically (equal-interval, equal-dose) derived
theophylline and regular beta-agonist aerosol bronchodilator medication
schedule constitutes evidence that asthma, while being primarily a
nocturnal disease, is not well-controlled by conventional treatment
schedules. The findings also are consistent with the hypothesis that a
greater amount of bronchodilator medication is required overnight than
during the daytime.

CHRONOTHERAPEUTICALLY OPTIMIZED THEOPHYLLINE THERAPY

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ABSTRACT

The clinical usefulness of a new chronotherapeutically-optimized, sustained-
release theophylline (SRT) formulation (BY158, TheoNite), administered once daily
in the evening for the management of asthma was evaluated. In a randomized, open
label crossover study, the administration of TheoNite at 8:00 p.m. was compared
with an equivalent daily dose of TheoDur divided equally for administrations at
8:00 a.m. and p.m. After a theophylline clearance determination to verify slow
or normal drug metabolism, a dose titration, and a 24-hour baseline spirometric
profile off all medication, the patients were randomized to a seven days of
either TheoNite or TheoDur therapy. A six day out-patient segment was followed
by a 24-hour inpatient study during which pharmacokinetic and pharmacodynamic
(including PEF and FEV_1) parameters were evaluated every two hours. The patients
were then crossed-over to the other theophylline preparation and the seven-day
treatment was repeated. When compared to baseline values, both SRT medications
significantly improved airflow over 24 hours. However, between 2:00 and 6:00
a.m., PEF and FEV_1 were significantly greater with TheoNite than TheoDur. In
conclusion, a chronotherapeutically-optimized, sustained-release theophylline
formulation, TheoNite, administered once daily in the evening was more
efficacious in controlling asthma over 24 hours than a conventional twice daily
agent.

KEYWORDS

Asthma, once daily theophylline therapy, chronotherapy

INTRODUCTION

Patients with asthma experience a worsening of symptoms at night with the
majority of dyspneic episodes occurring between 2:00 and 7:00 a.m. (Dethlefsen
and Repges, 1985). Turner-Warwick (1988) in a multicenter study evaluated 7661
patients with asthma by questionnaire and found that 39% awakened nightly and
64% awakened at least three nights a week despite the use of prescribed
bronchodilator medications. Current evidence shows that airflow obstruction
increases at night and in the early morning and is responsible for the
exacerbation in nocturnal symptoms (Hetzel and Clark, 1980). These symptoms
disturb sleep and are likely to disrupt daily performance. The development of
a therapeutic agent that better controls nighttime symptoms, without a worsening
of disease during the daytime, should improve the quality of life of many
asthmatic patients.

In the past, the treatment of asthma using sustained-release theophylline therapy has relied upon the administration of medications, generally twice daily using equal doses, so as to produce nearly constant blood levels throughout each 24 hours. The development of a drug delivery technology enabling the better control of theophylline blood levels represented a significant advancement in terms of efficacy and safety of drug dosing. However, this concept of theophylline therapeutics assumes that the airway requirement for therapy is the same nocturnally as it is diurnally. In certain, and perhaps most, asthmatic patients this therapeutic concept may not be valid; despite "adequate" serum theophylline levels measured during the day, asthma symptoms often worsen at night. From our prospective an ideal bronchodilator agent should have its maximal effect at the time of greatest bronchoconstriction.

The purpose of this study was to evaluate the clinical usefulness of a newly developed once-daily theophylline medication, TheoNite (Euphylong as trademark in Europe), formulated specifically to achieve an elevated and sustained serum theophylline level plateau overnight, especially between 2:00 and 6:00 a.m. (Benedikt et al, 1988; Steinijans et al, 1988). The efficacy of TheoNite was compared with a reference sustained-release theophylline, TheoDur, administered twice daily in order to determine the relationship between serum theophylline concentrations and change in airflow during the night as opposed to during the day.

METHODS

Twenty-five nonsmoking asthmatic patients between 20 and 60 years old were recruited for the study. All were being treated with a sustained-release theophylline preparation, had stable asthma and were diurnally active between 7:00 a.m. and 11:00 p.m. None of the patients had required either inhaled or systemic corticosteroid therapy within two months of the study. All had used one of several sympathomimetic inhalers as part of their treatment regimen. Each patient had to demonstrate at least a 15% improvement in FEV_1 after two puffs (131 mcg/puff) of inhaled isoproterenol. Only patients who were characterized by a clearance study as normal or slow theophylline metabolizers were included in the study. Of the initially enrolled 25 patients, 20 completed the study according to protocol. One was withdrawn because of failure to comply with the protocol. The remaining four patients required inhalations of isoproterenol during at least one of the three 24-hour study periods.

After qualifying as normal or slow theophylline metabolizers, patients were evaluated for their 24-hour baseline temporal pattern in pulmonary function. Theophylline medication was discontinued for at least 36 hours, and other bronchodilator medication at least eight hours, before initiating the baseline study. The spirometric indices of forced vital capacity (FVC), one-second forced expiratory volume (FEV_1), and peak expiratory flow (PEF) were determined every two hours starting at 9:00 p.m. during a single 24-hour period. Following this period, a theophylline dose titration period, using TheoDur, was performed.

Following dose titration, the patients were randomized into two groups. One group received TheoDur twice daily at 8:00 a.m. and 8:00 p.m. and the other received TheoNite once daily at 8:00 p.m. with a placebo at 8:00 a.m. Equivalent doses of theophylline were administered in each arm of the study. For seven consecutive days the patients took their theophylline treatment as specified, six days as outpatients and then as inpatients during the seventh day for measurements. During the inpatient 24-hour study period the patients were administered their theophylline medication and pulmonary function testing and blood sampling were done at two-hour intervals. Then, the groups were crossed-over from one theophylline treatment to the other, without a washout period.

Specific pharmacokinetic variables were analyzed for significant effect due to drug regimen by analysis of variance with subjects, drug regimen, and study period as the classification variables. Bioequivalence of the two theophyllines was assessed by the inclusion of the 90% confidence limits in the bioequivalence range of 80 to 120%. For each spirometric parameter, specific characteristics were compared between the baseline period and each treatment by the two-tailed Wilcoxon-Pratt Test, and between the two theophyllines by the distribution-free crossover analysis according to Koch.

RESULTS

Pharmacokinetics

The pharmacokinetic characteristics of TheoNite and TheoDur determined from their respective steady state 24-hour study periods are found in Table 1.

Table 1: Steady state pharmacokinetic characteristics for TheoNite and TheoDur during the 24-hour study periods (mean ± SD, n=24) * p<0.05

Characteristic	TheoNite o.d.	TheoDur b.i.d.
AUC_{ss} (mg/l.h)	232 ± 60	251 ± 59
C_{av} (mg/l)	9.7 ± 2.5	10.4 ± 2.5
C_{max} (mg/l)	13.7 ± 3.5	12.3 ± 3.0
t_{max} (h)	8 ± 2	12 ± 7*
C_{min} (mg/l)	5.2 ± 1.4	8.4 ± 2.3*
% peak-to-trough fluctuation	88 ± 12	37 ± 14*
% AUCF (%)	24 ± 4	8 ± 4*
Therapeutic occupancy time (h)	9.8 ± 5.9	12.6 ± 10.0
Plateau time (h)	10.8 ± 1.8	0.7 ± 3.9*
% nocturnal excess (%)	36 ± 8	3 ± 10*

TheoNite and TheoDur were equivalent with respect to the extent of drug absorption (AUC and C_{av}). For the pharmacokinetic parameters which characterize the intentionally different rate of absorption of TheoNite, the 90% confidence interval was not in the bioequivalence range with the exception of C_{max}. Also, the therapeutic occupancy time in the serum theophylline concentration range of 10-20 mg/l was not different. The t_{max} occurred between four and 10 hours after the evening dose of TheoNite and specifically at eight hours, i.e. at 4:00 a.m., in half of the patients. For TheoDur the t_{max} occurred randomly after drug intake, in some cases even just prior to the next dose apparently due to a lag in absorption. The percent nocturnal excess, a measure of the excess serum theophylline concentration between 2:00 and 6:00 a.m. with reference to the 24-hour average concentration, was 36 ± 8% for the TheoNite and 3 ± 10% for Theo-Dur.

Pharmacodynamics

When compared with baseline lung function profiles without medication, both theophylline treatments significantly improved the mesors, i.e. 24-hour average of all lung function parameters measured (Table 2).

Table 2: Lung function indices obtained during baseline and the two theophylline treatment periods (mean ± SD; n=20)

Lung Function Parameter	Baseline	TheoNite o.d.	TheoDur b.i.d.
PEF mesor (l/s)	5.1 ± 1.8	6.3 ± 2.4*	6.2 ± 2.3*
PEF_{av} (2:00-6:00 a.m.) (l/s)	4.5 ± 1.7	6.1 ± 2.2*⁺	5.5 ± 2.1*
PEF_{av} (2:00-6:00 p.m.) (l/s)	5.2 ± 1.8	6.4 ± 2.6*	6.5 ± 2.5*
FEV₁ mesor (l)	2.4 ± 0.9	2.8 ± 0.9*	2.8 ± 0.9*
FEV_{1av} (2:00-6:00 a.m.) (l)	2.2 ± 0.9	2.9 ± 1.0*⁺	2.6 ± 0.9*
FEV_{1av} (2:00-6:00 p.m.) (l)	2.5 ± 0.9	2.8 ± 0.8*	2.9 ± 0.9*

* p<0.05, baseline compared to each medication
+ p<0.05, TheoNite compared to TheoDur

The graphs of the 24-hour temporal pattern for PEF and FEV₁ are shown in the upper portions of Figure 1. None of the 24-hour averages for the pulmonary function parameters differed in a statistically significant manner between the two theophylline treatments. However, PEF and FEV₁ between 2:00 and 6:00 a.m. were significantly greater with TheoNite administered once daily in the evening than with twice daily TheoDur (p<0.05). It is of interest that there was no significant difference in either PEF or FEV₁ between the treatments twelve hours later during the afternoon, even though the average serum theophylline concentration for Theodur was somewhat greater than that for TheoNite.
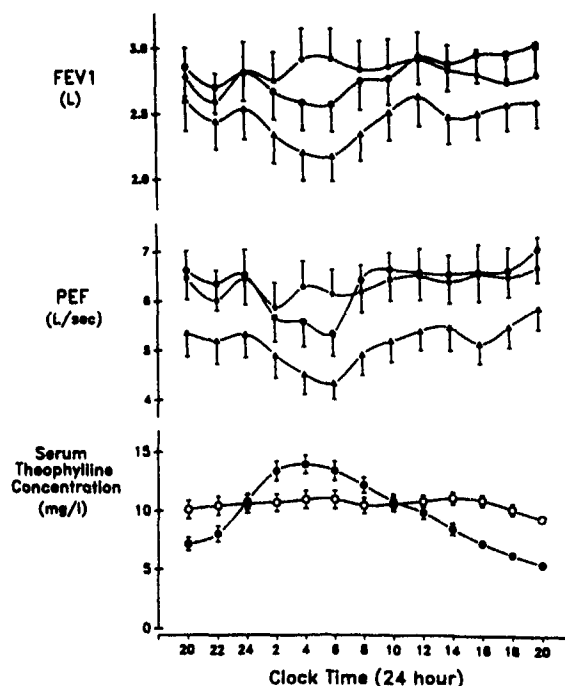
CONCLUSION

We conclude that for patients with asthma, especially those who suffer from
nocturnal symptoms, once daily TheoNite taken in the evening at 8:00 p.m. in
comparison to TheoDur taken twice daily results in a greater improvement in
airflow during the nocturnal period, when the risk of asthma is greatest. This
therapeutic advantage is achieved without a deterioration in airway status during
the daytime. Furthermore, even though the serum theophylline concentration from
the once daily administration of TheoNite exhibits greater variability during
the 24 hours in comparison to the twice daily administration of TheoDur, airway
status is more stable with TheoNite than TheoDur. Based on the findings of this
investigation on patients suffering from asthma, the once daily, evening
treatment in the form of TheoNite represents an optimization of theophylline
therapy in the treatment of asthma.

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THEOPHYLLINE THERAPEUTIC DRUG MONITORING IN THE CASE OF A NEW SUSTAINED-RELEASE PELLET FORMULATION FOR ONCE-DAILY EVENING ADMINISTRATION

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Summary: In view of the large interindividual differences in theophylline clearance and the narrow therapeutic range it is essential to individualize the theophylline dose. In order to do so, estimation of minimum and maximum serum theophylline concentrations during one dosing interval from one or two blood samples is desirable, particularly in the case of once-daily administration. Whereas the minimum (trough) concentration can be readily estimated from the pre-dose level, the maximum (peak) concentration occurs at night. For Euphylong°, a new sustained-release theophylline pellet formulation administered once daily in the evening, the nocturnal maximum concentration can be calculated as 110-120% of the serum theophylline concentration determined from a blood sample taken in the early morning, for example between 7 a.m. and 8 a.m. This procedure works not only for mean data but also on an individual basis. The procedure is based on the extended nocturnal plateau profile of Euphylong with its high reproducibility and cannot be transferred to other formulations.

Introduction: Although therapeutic drug monitoring is generally recommended for the optimizing of theophylline therapy, information about the pharmacokinetic profile is lacking for many formulations.

Unless the time of the last dose and the pharmacokinetic profile of the administered formulation is precisely known, completely erroneous conclusions may be drawn. The situation becomes even mor complicated if pronounced day-and-night differences (1, 2) or pronounced administration-time dependencies (3) are observed. It is the merit of the chronobiologists that they penetrated the problem of the substantially different information content obtained at different sampling times both in relation to drug intake and day and night rhythms (4, 5). In doing so, they showed that, at least for certain formulations, one or two sampling times are optimal for therapeutic drug monitoring.

Secondly, dry reagent technology has provided quick and easy-to-use methods for quantitative determination of theophylline in serum, such as the Seralyzer°(Ames Division, Miles Laboratories, Inc., Elkhart, IN, USA) and the AccuLevel° Theophylline Test (Syntex Medical Diagnostics, Div. of Syva Co., c/o Data Type, New York, NY,

USA).

It is the purpose of this paper to show that the pharmacokinetic
profile of Euphylong pellets allows a simple and reliable method
for individually estimating the nocturnal maximum concentration
from an early morning blood sample taken during clinical routine.
Materials and Methods: Let Cmax and Cmin denote the maximum (peak)
and minimum (trough) serum theophylline concentration (STC) observ-
ed during a 24-h dose interval in steady-state; moreover, let Ct
denote the STC at time t, t being the clock time given in military
notation, e.g. C0800 denotes the STC at 08.00 h or 8 a.m., and
C2000 denotes the STC at 20.00 h or 8 p.m. For each time t during
the 24-h dosing cycle, the per cent error in underestimating Cmax
is then given by: %Error in underestimating Cmax=100 (Cmax-Ct)/
Cmax.

By definition, all concentrations Ct are less than or equal to Cmax
so that the per cent error in underestimating Cmax is already ex-
pressed as positive value. In the original definition (6), the sign
was just the other way round and hence negative.

The above procedure is illustrated by Fig. 1 and Table 1, which
give the steady-state plasma theophylline concentrations of Subject
11 on day 5/6 in a multiple-dose bioavailability study (7). Subject
11 (male, 25 years, 85 kg, 197 cm) received 9.4 mg/kg/d theophyl-
line as Euphylong pellets. It is seen from Fig. 1 that the pre-dose
level at 20.00 h (8 p.m.) corresponds to the minimum concentration:
day 5: C2000=9.40 mg/l; day 6: C2000=9.02 mg/l.

The maximum concentration occurs at 2 a.m. and is followed by a
flat plateau overnight, which begins to decline after 8 a.m.

Table 1 shows that in this subject the per cent error in underesti-
mating Cmax is 4% at 6 a.m. and 15% at 10 a.m.

The procedure described above is carried out for each time t during
the 24-h dosing interval and for each subject in the study. The
values are then tabulated. Mean and SEM are given as summary statis-
tics.

Results: Fig. 2 shows the individual values of the per cent error
in underestimating Cmax at each time of the 16 measurements during
the 24-h dose interval on day 7/8 in a multiple-dose, randomized,
crossover study comparing Euphylong pellets with a reference formu-
lation (Reference A, two 400 mg tablets) in healthy volunteers (6).
In order to match the dosage strength of the reference product,
capsules containing 400 mg anhydrous theophylline as Euphylong pel-
lets were used.

The evening dose of 10.8 mg/kg/d was administered at 7 p.m., half
an hour after a standardized evening meal. The maximum concentra-
tion was 13.4 ± 2.9 mg/l (mean ± SD, n=12) for Euphylong pellets
and occurred 8 (5-14) h after dowing (median and range); the cor-
responding values for Reference A were 17.8 ± 4.3 mg/l (p<0.001)
for Cmax and 6 (5-14) h after dosing for Tmax. The range of Tmax
can also be seen in Fig. 2 from the open and closed circles on the
x-axis which correspond to zero error in underestimating Cmax; in
other words, these values reflect Cmax.

The reproducibility of the nocturnal plateau profile between sub-
jects and within subjects from day to day has been described in a
previous publication on the pharmacokinetic profile of Euphylong
pellets (8). Fig. 3 shows mean ± SEM (n=18) of the per cent error
in underestimating Cmax for two 24-h dose intervals, namely day 5/6
and day 8/9, in the same group of subjects (7). In this study, the
evening dose of 11.8 mg/kg/d was administered at 8 p.m., half an
hour after a standardized evening meal. The maximum concentration
was 12.7 ± 3.1 mg/l (mean ± SD, n=18) on day 5/6 and occurred
6 (5-12) h after dosing (median and range); the corresponding
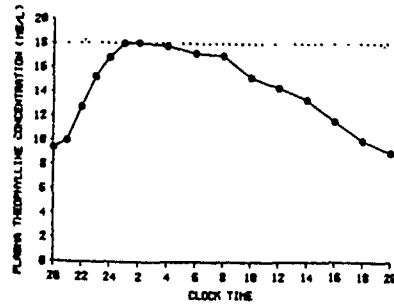
Fig. 1: Plasma theophylline concentrations of subject 11 following repeated evening administration of 9.4 mg/kg/d theophylline as Euphylong pellets. The dotted line indicates the maximum concentration of 18 mg/l which occurred at 2 a.m

Table 1: Plasma theophylline concentrations (PTC) and per cent error in underestimating C_{max} during a 24-h dose interval in steady state (Subject 11, day 5/6, reference [7])

Time after last dose (h)	Clock time	PTC (mg/l)	Per cent error in underestimating C_{max}
0	8 p m	9 40	48
1	9 p m	10 01	44
2	10 p.m	12.75	29
3	11 p.m.	15 23	15
4	12 midnight	16 81	7
5	1 a m	17 97	0
6	2 a.m	18 00	0
8	4 a m.	17 77	1
10	6 a m	17 21	4
12	8 a.m	17 01	6
14	10 a m	15 23	15
16	12 noon	14 40	20
18	2 p m	13 40	26
20	4 p m	11.61	36
22	6 p m	9.99	45
24	8 p m	9 02	50
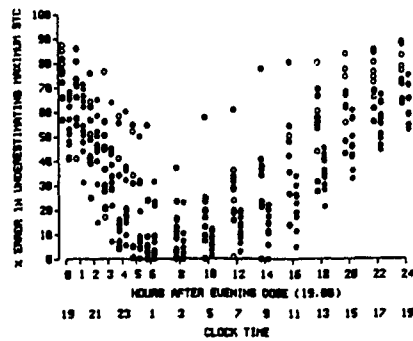
Fig. 2: Individual values of the per cent error in underestimating C_{max} at all times of measurements during a 24-h dose interval in steady-state (● = Euphylong pellets, O = Reference A).
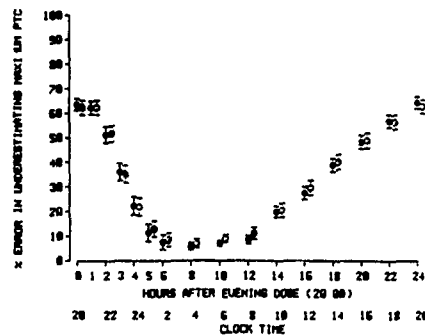
Fig. 3: Mean ± SEM, n = 18 of the per cent error in underestimating C_{max} at all times of measurements during two 24-h dose intervals in steady-state (● = day 5/6, O = day 8/9) The evening dose was 11 8 mg/kg/d theophylline as Euphylong pellets

values on day 8/9 were 13.1 ± 3.0 mg/l and 7 (4-14) h after dosing.
At 8 a.m. the per cent error in underestimating Cmax was 9 (0-20)%
on day 5/6 (mean and range) and 11 (0-36)% on day 8/9. At 10 a.m.
the error had increased to 20 (1-34)% and 21 (0-49)% on day 5/6 and
day 8/9, respectively. Therefore, it is recommended to draw the
blood sample for estimation of Cmax preferably at 8 a.m. rather
than at 10 a.m. Generally speaking, the sample should be drawn 12h
after the evening dose.
Finally, the method for therapeutic drug monitoring of once-daily
Euphylong pellets has been applied in a prospective manner. To this
end, the plasma theophylline concentration at 8 a.m. on day 6 was
used to individually predict the nocturnal maximum concentration
at day 9. The predicted maximum was then compared with the measur-
ed maximum on day 9 in the same subject. Fig. 4 shows the correla-
tion between the actually measured Cmax-values on day 9 (y-axis)
and the predicted Cmax-values (x-axis). The predicted Cmax-values,
Cmax, were calculated on the basis of the 8 a.m. concentration on
day 6 by means of the following formula: $Cmax=1.15 \, C_{0800}$; this
means that the nocturnal maximum concentration was assumed to be
15% higher than the measured concentration at 8 a.m. Moreover, it
was assumed that the concentration/time profiles on days 5/6 and
8/9 were reproducible to such an extent that the Cmax-values were
practically identical. The coefficient of correlation was r=0.703
(p < 0.001, one-sided). In view of the known day-today variability
of theophylline pharmacokinetics, in particular the clearance,
this result is very satisfactory.
Discussion and Conclusion: Therapeutic drug monitoring is a re-
latively new and rapidly developing medical technology. In the
leading article on "Cost-Effectiveness of Therapeutic Drug Moni-
toring" (9), Vozeh stated that "an effective strategy should in-
clude, in addition to a reliable and rapid drug assay, recommenda-
tions for optimal sampling times and and efficient algorithm for
predicting individual pharmacokinetics. To be applicable in the
clinical routine, the strategy has to be realistic and simple to
follow."
For once-daily Euphylong pellets such a simple method of therapeu-
tic drug monitoring has been described. Attention has been focused
on estimating the maximum concentration during a 24-h dose inter-
val, as theophylline dose titration is usually done in an upward
fashion and as the maximum concentration is the criterion for dose
increase. Utilizing the knowledge about the entire 24-h concentra-
tion/time profile, a once-point method has been suggested, which
needs only a simple calculation and not a complicated algorithm.
This, in combination with a reliable, quantitative on-the-spot
assay, may contribute to optimize theophylline therapy to the bene-
fit of the individual patient.

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PRESERVATION OF THE ADRENAL FUNCTION WITH ORAL MORNING DOSING OF CORTICOIDS IN LONG TERM (3 TO 11 YEARS) TREATED CORTICO-DEPENDENT ASTHMATICS

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ABSTRACT

In nine patients suffering from a nocturnal cortico-dependent asthma
the long term chronotherapy with morning dosing of 10 to 50 mg
prednisolone (or prednisone) was associated with 1/ no adrenal
suppression : persistence of both plasma cortisol circadian rhythm
and responsiveness to ACTH stimulation and 2/ no osteopenia observed
in 6/9 patients as compared to the occurrence of osteopenia in 18/30
patients treated with oral corticoids not restricted to morning
dosing (P < 0.04).

KEYWORDS :

Plasma cortisol; ACTH test; Asthmatics; Osteopenia; Corticotherapy.

BACKGROUND

In diurnally active patients, the morning dosing of corticoids
(equivalent to ~ 15 mg prednisone 24 h) does not induce adrenal
suppression, even after one month. (Reinberg et al 1977, 1983, 1988,
1989). Is this valid for cortico-dependent asthmatics treated for
many years since adrenal suppression appears to be the major
undesired effect? Another major undesired effect is the risk of
osteopenia. In our first attempt to answer these questions
(Reinberg, Touitou et al, 1988) the plasma cortisol circadian rhythm
of long term treated asthmatics (10 to 50 mg/24 h prednisone or
prednisolone with dosing time exclusively at 08 h) was documented
before and during an ACTH test. A RIA method was used for
determinations. In principle, it does not provide the expected
results since the contribution of metabolites of prednisolone might
alter the cortisol determination.

AIMS

Aims were : 1/ to test the validity of determinations obtained with
RIA method using two other methods : the FPIA (TDX,Abbott) method
which is both sensitive and rather specific, and the HPLC method
which is less sensitive than either RIA or TDX.

        2/ to document the vertebral bone density in 9 (rather
than 6) subjects with morning dosing of oral corticoids for 3 to 11
years.

        PATIENTS

Nine diurnally active patients with nocturnal asthma volunteered for
the study : 2 women (26 and 46 years of age) and 7 men (36 to 53
years of age). The morning dosing of corticoids (either prednisone
or prednisolone or both) ranged from 5 to 30 and 20 to 50 mg/24 h.
The duration of this chronotherapy ranged between 3 and 11 years.
One month prior to data gathering the treatment was standardized on
20 mg prednisolone at 0800 (Solupred R Houdé, France).

        METHODS

- CORTISOL CIRCADIAN RHYTHM AND ACTH TEST. During a 48 h span,
starting at 0800 blood was sampled every 3 hours to document changes
in plasma total endogenous cortisol before (24 h) and after (24 h) a
subcutaneous injection (at 0800) of 10 μg ACTH-1-17 (Synchrodyn
Hoechst) to test the adrenocortex responsveness.

The FPIA-TDX method was used since the cross-reaction of
prednisolone was then reduced from 70% (RIA) to 20% with regard to
plasma cortisol. Plasma samples of 3 additional patients were
processed together with those of 6 patients studied previously
(aliquots of their plasma had been preserved at -80°C).

- BONE DENSITOMETRY. A monoenergetic scanner-base method to quantify
vertebral bone density (so-called bone densitometry) was used to
estimate osteopenia taking into account age, sex, as well as
ethnical origin of the patients with regard to a pertinent reference
scale

        RESULTS

Observed changes in plasma cortisol with data obtained using 3
different methods of determination (RIA, FPIA-TDX and HPLC)
exhibited only rather small differences (figures 1 and 2). However,
as compared to the TDX method, data were overestimated with RIA
method and underestimated with the HPLC method, especially around
peak time, when plasma cortisol concentration is high.
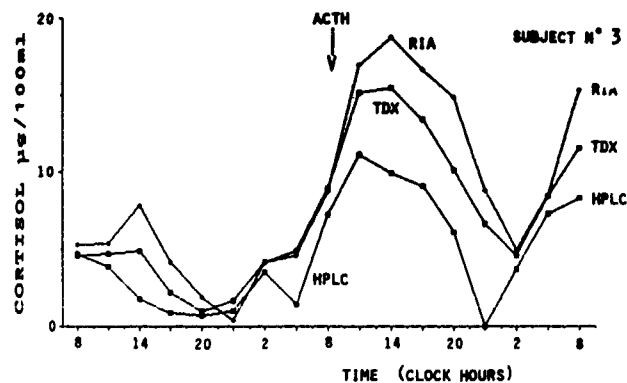
Figure 1
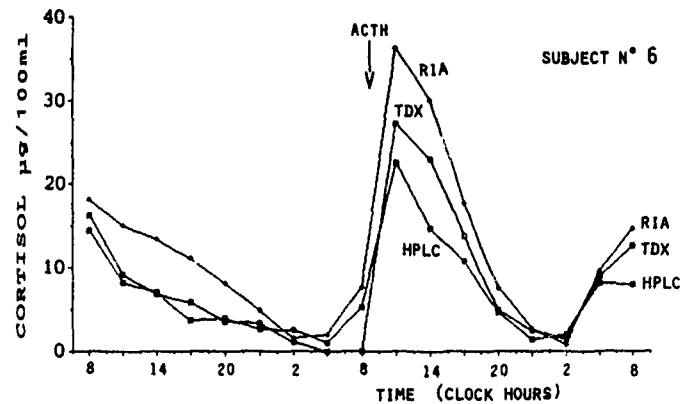
Figure 2

In all patients a large amplitude (peak to trough ratio = 11.5±1.8) circadian rhythm of plasma cortisol (TDX), with a peak (10.8±1.7 μg/100 ml) occurring in the morning hours (09.42 a.m.±90 mn) and a nocturnal trough (from 0.3 to 1.9 μg/100 ml) was observed before ACTH injection. The ACTH induced increase of the plasma hormone (24 h AUC as % of control) ranged from + 20 to + 171% (mean 88%±27) (Table 1). This result fits well with data obtained by Angeli et al (1981) who found that 10 g of ACTH 1-17, injected IM at 08 h to healthy adults induced a 98%±49 increase of AUC with regard to control saline (recomputed from published data).
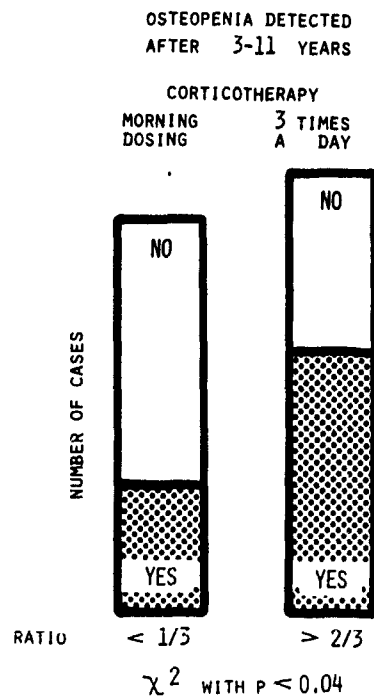
Figure 3

No osteopenia was observed in 6/9 patients. Subjects 2 and 4 had osteopenia while subject 8 was borderline . In comparison, using the same method, osteopenia was observed in 18 out of 30 patients treated with oral corticoids not restricted to morning dosing. The difference between these two groups is statistically significant : χ 2 = 6.3 with P<0.04 (figure 3).

TABLE 1: PLASMA CORTISOL

Subject's number	Morning peak height ug/100ml Ø:clock hours		Amplitude peak/trough ratio	ACTH-induced increase of 24h AUC
1	6.3	14h	21	+ 34%
2	13.7	8h	9.8	+164%
3	8.9	8h	8.9	+161%
4	11.6	8h	13.9	---
5	16.2	8h	14.7	+171%
6	4.0	11h	6.7	+ 20%
7	4.8	14h	8.0	---
8	13.5	8h	5.6	+ 31%
9	17.9	8h	9.4	+ 33%
X̄	10.8	9.7	11.5	+ 88%
+ SEM	±1.7	±1.5	±1.8	±27%

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CIRCADIAN CHANGES IN THE SIZE OF FACIAL SKIN CORNEOCYTES OF HEALTHY WOMEN

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ABSTRACT

Circadian changes in the size of facial skin corneocytes of 24 healthy women has been demonstrated, with a peak time at 2300 h and a trough at 1100 h. With regard to both age and time of day small size corneocytes appear to be related to a better appearance of the facial skin than those of large size.

KEYWORDS

Circadian rhythm; Corneocytes; Facial skin appearance.

BACKGROUND

In a first study we reported time-related changes in self-rated items of facial skin appearance (1).

AIM

The aim of this study was to document time-related differences in the size of facial skin corneocytes. Stratum corneum is th first protective layer (12-15 μm thick) of the skin; it also acts as a barrier to the unrestrained passage of water in either direction across the skin. It is composed of overlapping, thin, disk-like corneocytes.

SUBJECTS

Age	Number of subjects in each subgroup.
20-30	4
31-40	12
41-50	4
> 50	4

METHODS

Corneocytes were removed using adhesive tape and their mean size quantified thereafter. The observed area was expressed in   m2. During a 48h span, at fixed clock hours 0730, 1100, 1930 and 2300 h

corneocyte- were sampled, and a set of variables were self-rated as
well. Visual analog scales (horizontal rectangles 22 mm wide and 5
mm high), allowing a quantification from 0 to 22 in arbitrary units,
were used to self rate 5 items by the placement of a vertical pencil
stroke positioned as an index. The further to the right, the higher
the score rated.
The following items were evaluated :
1/ with regard to facial skin :
. Texture of the skin (extremes : from very smooth to very rough).
. Brilliance of complexion (extremes : from pale to glowing).
. Appearance of facial skin (extreme) : from weary to fresh).
. Overall impression (extremes : from poor to excellent).
2/ With regard to subject behaviour :
. Fatigue (extremes : from exhausted to full of pep).
. Mood (extremes : from very bad to very good).
Visual analogue scales and self-ratings are commonly used in
chronopharmacology to document circadian rhythms of both fatigue and
mood, among many other variables, e.g. pain, dyspnea, drowsiness,
etc.
Several statistical methods were selected to analyze the time series
thus collected. First, data were visualized mainly by the plexogram,
i.e. mean of each variable at each time point of measurement in the
24 hr scale, for each experimental situation as well as each group
and/or subgroup. Any time point mean is given with its standard
error : X̄±1 SEM (cf. Figure 2). An ANOVA test was used to validate
significance of observed differences related to time of day, time of
treatment as well as age class and complexion of skin.
The cosinor method was used mainly to estimate the 24-hr adjusted
mean M±1SEM (Cf. Figure 1).
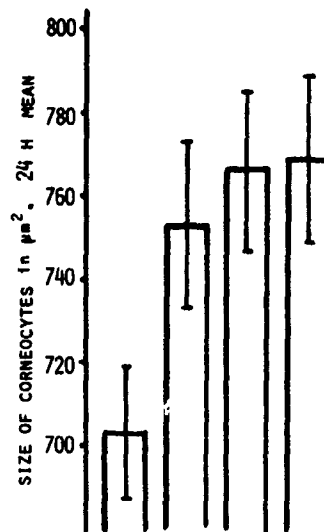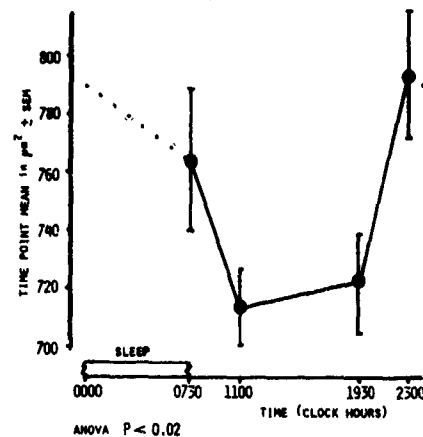
Figure 1

Figure 2

RESULTS

AGE-RELATED CHANGES IN SIZE OF CORNEOCYTES HAVE BEEN VALIDATED (FIGURE 1).

The smaller area (X = 703 µm2+(SEM) 16) corresponds to younger subjects and the greater (X = 769 µm2 + 20) to older ones.

TIME-RELATED CHANGES IN SIZE OF CORNEOCYTES HAVE BEEN VALIDATED AS WELL.

Taking into account all subjects (Figure 2) the peak time occurred at 2300 h (X = 794±22 µm2) and the trough at 1100 h (X=714±17 m2). In fact, low values were obtained at 1100 and 1930 h. No statistically significant change in size of corneocytes were observed with regard to hue of skin or hair (Figure 3).

TIME-RELATED CHANGES HAVE BEEN VALIDATED IN SELF-RATED VARIABLES:

Texture of skin, brilliance of complexion, appearance of facial skin, overall impression as well as mood (Figure 4). Peak times occurred either at 1100 h or 1930 h while trough time was always 0730 h.

THE CIRCADIAN RHYTHM OF SELF-RATED FATIGUE APPEARED TO BE AGE-RELATED (FIGURE 5).

NO CHANGE IN SIZE OF CORNEOCYTES
WITH REGARD TO HUE OF SKIN AND HAIR

ANOVA, TIME-RELATED DIFFERENCES P< 0.04
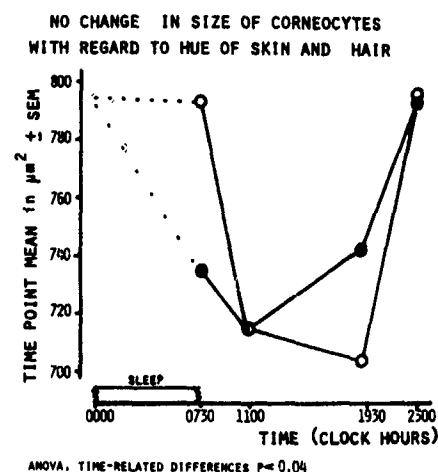
0 = Fair complexion; ● = Dark complexion

Figure 3

TIME-RELATED CHANGES (RAW DATA) WITH REGARD TO SELF-RATED VARIABLES

TEXTURE OF FACIAL SKIN

BRILLIANCE OF COMPLEXION

APPEARANCE OF FACIAL SKIN

OVERALL IMPRESSION

MOOD

ANOVA FROM P<0.006 TO P<0.0000
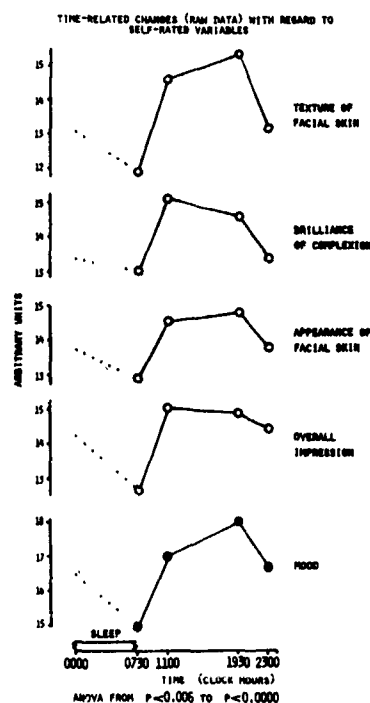
Figure 4

AGE RELATED DIFFERENCE IN CIRCADIAN RHYTHM
OF SELF-RATED FATIGUE
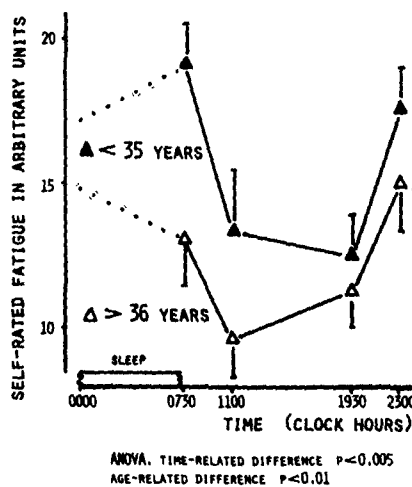
Figure 5

COMMENTS

Circadian changes in size of corneocytes is an intriguing
phenomenon since these cells pertain to the complex structure of
the stratum corneum which is permanently renewed. The discussion of
possible mechanisms would be purely academic at this stage of
investigation.

With regard to both age and time of day small size corneocytes
appear to be related to a better appearance of the facial skin than
those of large size. Thus, size of corneocytes would be one the
factors (among others to be found) which contribute to periodic and
predictable changes in the aspect of facial skin.

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M., Smolensky M. : Chronobiol. Internat. : 7; 1990 (in press).

CIRCADIAN CHARACTERISTICS OF SEVERAL URINARY VARIABLES FOLLOWING MORNING EDTA INFUSION: A CASE REPORT

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INTRODUCTION

The use of EDTA salts for the removal of heavy toxic metals, particularly lead, from the bloodstream, as well as in cardiovascular chemotherapy has been amply documented and reviewed (Harper, 1976). Its usefulness in removing toxic levels of lead is widely accepted, however its therapeutic value in cardiovascular disease, although acclaimed by some physicians (Casdorph, 1981; Cranton, 1984), has been questioned by others (Harvard, 1984; Casdorph, 1988; McGillem, 1988). The present controversy over the administration of EDTA chelation therapy for purposes other than the removal of lead prompted us to reexamine a number of urine variables (pH, total solids, total proteins, Na, K, Ca, Mg and Zn) in order to study the circadian response to its infusion.

SUBJECT, MATERIALS and METHODS

Subject The subject was a 65-year-old male (ELK) who was clinically symptom free. His diet consisted of low salt, low cholesterol foods with meals at 0715 and 1715 during the course of this study. His activities were routine as a hospital staff member and sleeping habits were normal, with lights out at 2200 and on at 0600, except for disruption due to urine collections at 0100 and 0400.

Material The infusion solution consisted of 3gm edetate disodium USP and 4ml of 50% magnesium sulfate injection in 500ml of lactated Ringer's USP (Travenol) containing 1680 mg Na, 404 mg Mg, 79 mg K and 36 mg Ca.

Methods The 4-hr EDTA infusion was performed in an office setting starting at 1000 and ending at 1400 on Aug 31, Sept 14, 28, Oct 5 and Oct 21, 1987. Control urines were collected on Mar 23, July 27, Oct 12 and Dec 28. On each of these days urines were collected, measured and aliquoted at 3-hr intervals. After the Oct 21 infusion, the 3-hr urine collections were continued for 4 additional days with a 24h collection on Oct 26. The urines were collected in polyethylene bottles and frozen. Each urine was measured for volume, pH, and analyzed for the following variables: total solids, Na, K, Ca, Mg and Zn using conventional methods of analysis. Total proteins were determined using the Coomassie Brilliant Blue G-250 stain. Values were expressed as rate/3h and ratios were calculated for Na/K and Ca/Mg. Circadian variation of each 24h profile was assessed by the range of change (ROC = difference from lowest to highest value). EDTA and control data were analyzed for circadian rhythm by the least-squares fit of a 24h cosine (Nelson, 1979) and rhythm characteristics were compared by the Bingham test of rhythm parameters (1982). No data were excluded or filtered.

RESULTS and DISCUSSION

Results Circadian characteristics of data obtained for the control periods and for the periods following EDTA infusion are presented in Table 1, while mean waveforms are shown in Fig 1 for

TABLE 1: Circadian Rhythm Characteristics After Days on EDTA vs Control Days

Variable	units	EDTA (n = 40 samples)				Control (n = 32 samples)			
		M±se	A±se	Ø	(95% limits)	M±se	A±se	Ø	(95% limits)
Ca	mg	53.9±5.6*	58.3±7.9*	1400	(1300,1500)*	21.9±1.1	5.7±1.6	2316	(2108,0124)
Mg	mg	27.7±1.6*	3.6±2.3	1016	(-)	15.6±0.8	3.2±1.1	0040	(2208,0316)
Ca/Mg	ratio	1.8±0.1*	1.8±0.2*	1436	(1400,1516)	1.4±0.1	0.2±0.1	2000	(-)
Na	mg	433±22	101±31	1432	(1216,1652)*	373±24	103±34	2344	(2116,0216)
K	mg	242±11*	142±16	1356	(1308,1448)	294±21	124±30	1320	(1132,1508)
Na/K	ratio	2.0±0.1*	0.8±0.1	0140	(0036,0248)	1.5±0.1	1.0±0.1	0128	(0048,0204)
pH		6.3±0.1*	0.4±0.1	1740	(1544,1948)*	5.9±0.1	0.3±0.1	0436	(0200,0716)
Protein	mg	11.5±1.0	5.4±1.5	1800	(1556,2000)	11.8±1.5	6.4±2.2	1828	(1556,2100)
Solids	gm	6.0±0.2	1.1±0.3	1440	(1252,1640)	5.5±0.2	0.5±0.2	2204	(-)
Volume	ml	222±18	33±25	1700	(-)	247±20	37±29	0916	(-)
Zn	µg	3207±234*	3337±331*	1608	(1524,1652)	95±5	8±6	0356	(-)

(M=MESOR, A=Amplitude; se = standard error; Ø = acrophase [ref: 0000]; 95% limits for Ø given if p≤0.05 from zero-amplitude test; *indicates significant difference at p≤0.05 by parameter test)

those variables in which significant alterations in rhythm parameters could be statistically confirmed. Considerable differences in the Mesor, amplitude and acrophase of variables between the control and EDTA periods can be attributed to the effects of EDTA. During the 24h following EDTA infusion a statistically-significant increase in 24h MESOR was found for Ca, Mg, Ca/Mg, Na/K, pH and Zn, while a significant decrease was found for K. Amplitude increases were observed in Ca, Ca/Mg and Zn, while shifts in Ø were found in Ca, Mg, Na and pH. A profound change was observed in the Mesor for Zn: during the first 24h after infusion there was a 33-fold increase in the excretion of Zn over the control level. There was also a 2.5-fold increase in the excretion of Ca and a 1.8-fold increase in the excretion of Mg. It seems likely that the increase in Zn was derived from the blood rich carbonic anhydrase (Maren, 1967). The Ca was probably derived, in part, from blood Ca, since serum concentrations are reduced by EDTA (3h sampling of Ca [mg/dl]: 9.7, 9.0, 8.8, 8.6, 9.3, 9.5, 10.1, 9.9, 10.0, 10.0 [Kanabrocki, 1981]). However, when these variables are examined in subsequent circadian collections, the rhythm characteristics for pH, Na, K, Ca and Mg return to control levels, indicating a transitory, masking effect of EDTA (Figs 2-3).
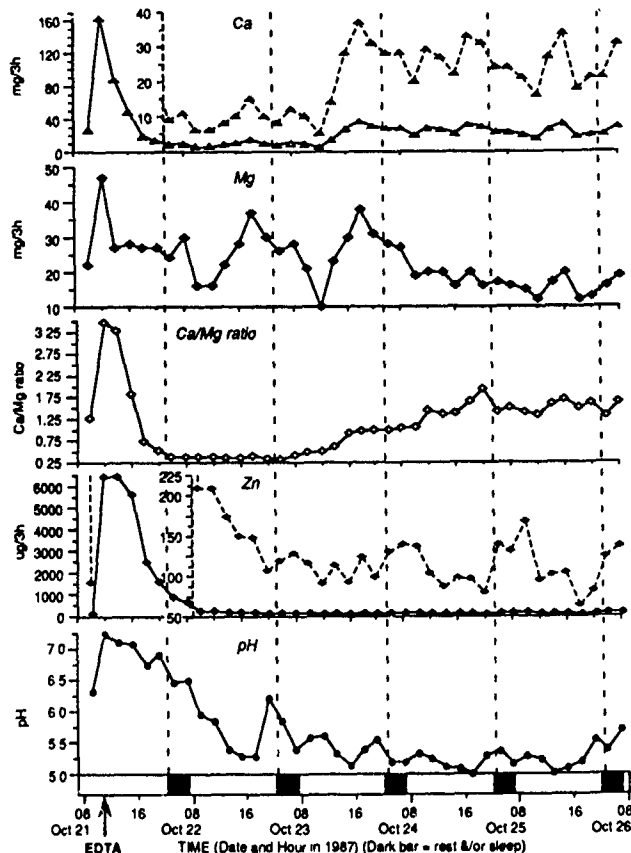
Fig 2: Urinary Excretion Rates Over Several Days Following Morning EDTA Infusion
EDTA administered between 1000-1400 (arrow) by Man (EK, age 65y)
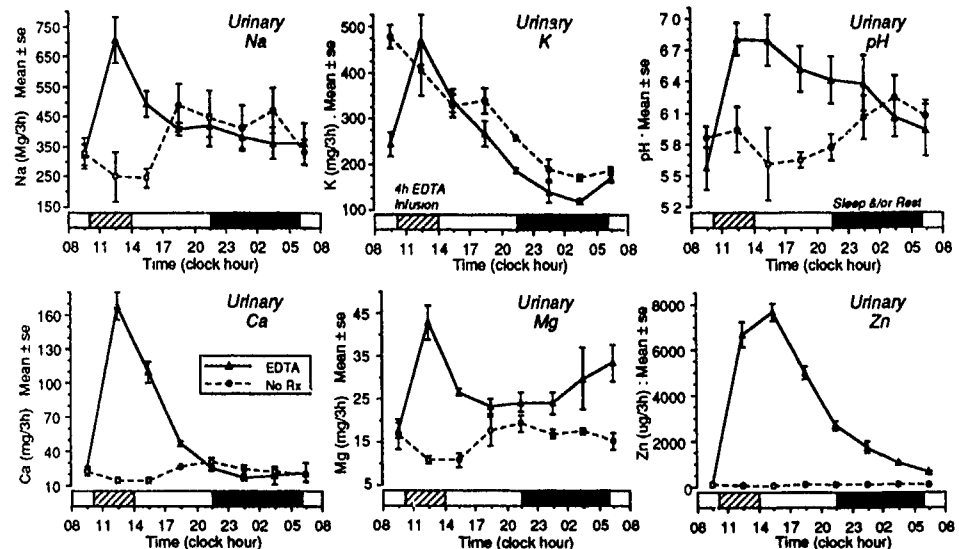
Fig 1: Circadian Variation in Urinary Excretions Following Morning EDTA Infusion
(Means from 4 control days vs 5 days following EDTA between 1000-1400 in a man [EK, age 65y])
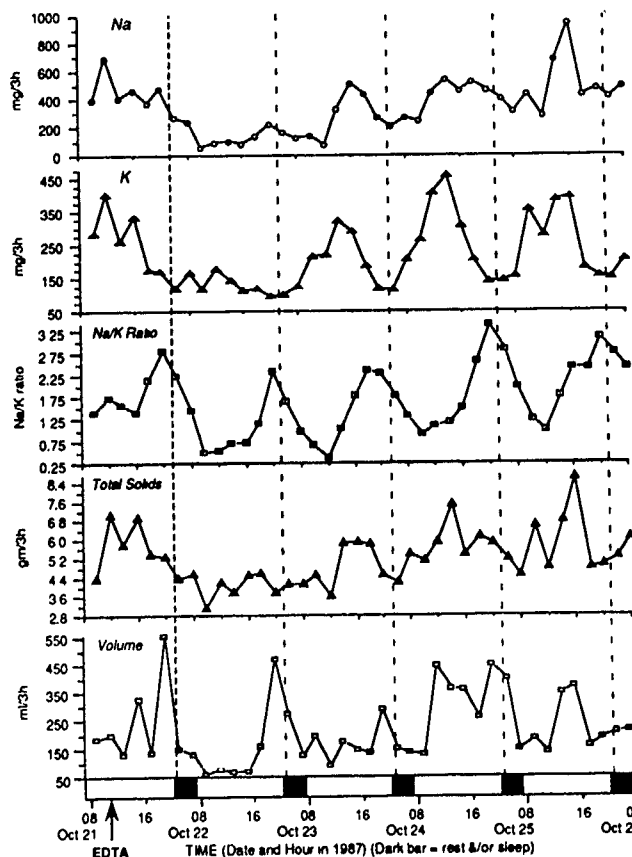
Fig 3: Urinary Excretion Rates Over Several Days Following Morning EDTA Infusion
EDTA administered between 1000-1400 (arrow) by Man (EK, age 65y)

Discussion The assessment of the effects of EDTA on urine excretion of electrolytes must be made with consideration of their amounts present in the solution infused (Na: 1680 mg; K: 79 mg; Ca: 36 mg; Mg: 404mg). It appears that the 24h Na and K levels were decreased during the second day after infusion and return to control levels on subsequent days (Table 2). After EDTA infusion, the Ca excretion was increased 2.5 times over the control level (431 vs 175 mg/24h). The increase in the Mg level observed in the first 24h period after infusion (221 v 125 mg) represents a portion of the amount actually infused. This increase continues to be evident through the following 3 days, accounting for nearly the entire amount of Mg infused. It may be speculated that the additional Mg present in the EDTA preparation was absorbed by the intra-cellular Mg-stores in the body and slowly released during subsequent days after infusion (Table 2). This phenomenon of slow release of Mg may be related to the reported clinical

Table 2: 24h total urinary excretion (mg) after EDTA infusion (Day 1)

Variable	24h spans after EDTA infusion:					
	1	2	3	4	5	6
Na	3464	983	2249	3403	4166	2451
K	1936	991	1671	2080	2104	1973
Ca	395	75	181	210	179	199
Mg	221	203	208	144	124	127
Ca/Mg	-	.36	.87	1.45	1.44	1.56

improvements observed in cardiovascular patients receiving Mg (Rasmussen, 1986; Smith, 1986) and warrents additional studies. The urine Ca/Mg ratio may prove to be a valuable tool in monitoring and assessing the effects of chelation therapy. Scrutiny of the effects of chelation therapy at other circadian stages is warrented.

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Annual Review of
CHRONOPHARMACOLOGY

Editors: A REINBERG · M SMOLENSKY · G LABRECQUE

Volume 7

BIOLOGICAL RHYTHMS AND MEDICATIONS

*Proceedings of the Fourth Conference of Chronopharmacology,
Nice, France, 12–15 March 1990*

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Endocrinology and Gastroenterology
   Chairpersons — Y. Touitou and E. Haus

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